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(54) Title: NUCLEIC ACID ENCODING A NOVEL CHEMOTHERAPY-INDUCED PROTEIN, AND METHODS OF USE																				
(57) Abstract <p>An isolated nucleic acid molecule is provided which encodes a mammalian signal transducing protein (Killer) involved in the induction of apoptosis. The encoded protein comprises an amino-terminal extracellular domain, a hydrophobic transmembrane domain, and a carboxy-terminal death domain that can form protein-protein interactions with other death domain-containing proteins to transmit the signals required for apoptosis. The invention also provides the Killer protein and antibodies thereto. These biological molecules are useful as diagnostic and therapeutic agents for the identification, detection and regulation of complex signaling events leading to apoptotic death. The molecules may also be used to advantage for assessing the potential chemosensitivity of a targeted tumor cell. Additionally, these molecules may be used as research tools to facilitate the elucidation of the mechanistic action of the Killer proteins of the invention.</p>																				

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**NUCLEIC ACID ENCODING A NOVEL CHEMOTHERAPY-INDUCED
PROTEIN, AND METHODS OF USE**

This application claims priority to the following United States Provisional Applications, all of which are incorporated by reference herein: 60/052,305, filed July 11, 1997; 60/054,710, filed August 4, 1997; 5 60/060,473, filed September 30, 1997; 60/077,661, filed March 11, 1998; 60/077,628, filed March 11, 1998; and 60/077,526, filed March 11, 1998.

FIELD OF THE INVENTION

10 This invention relates to diagnosis and treatment of neoplastic diseases. More specifically, this invention provides novel nucleic acid molecules, proteins and antibodies useful for detection and/or regulation of neoplastic cellular changes.

15

BACKGROUND OF THE INVENTION

Several publications are referenced in this application to more fully describe the state of the art to which this invention pertains. The disclosure of each 20 such publication is incorporated by reference herein.

The p53 tumor suppressor protein is a nuclear phosphoprotein that functions in cell-cycle arrest, programmed cell death (apoptosis), inhibition of tumor growth, and preservation of genetic stability. It 25 performs these functions through involvement in several biochemical pathways, including transcriptional activation, transcriptional suppression and inhibition of DNA replication. Loss of proper p53 function in cells is one step in the progression toward a neoplastic 30 phenotype; more than 50% of human cancers have mutations in the p53 gene (see V.E. Velculescu & W.S. El-Deiry, Clin. Chem., 42:858-868, 1996; A.J. Levine, Cell 88: 321-331, 1997).

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DNA binding and transcriptional activation are among the best-understood functions of p53. Nearly every tumor-derived p53 mutant has lost its ability to bind DNA and transcriptionally activate nearby genes. This
5 observation suggests that these properties of p53 are critical to its role in the control of cell proliferation. The diverse nature of the genes that p53 transcriptionally regulates suggests that p53 may be involved in pathways of cell-cycle control, angiogenesis,
10 DNA repair, differentiation, growth factor signaling and apoptosis.

Experimental evidence indicates that p53 activates a cell suicide pathway (apoptosis) after treatment with chemo- or radiotherapy. In some cell
15 types, the cytotoxic action of anti-cancer agents may be mediated through p53-dependent apoptosis. p53 regulates a number of genes involved in apoptosis, including *bax*, *bcl-2*, insulin-like growth-factor binding protein-3 (*igf-bp3*), and *fas*. The genes for Bax and IGF-BP3 have been
20 shown to contain p53-dependent, *cis*-acting, DNA-responsive elements.

Inactivation or mutation of p53 has been reported to enhance cell sensitivity to cytotoxic agents which induce DNA damage. However, the status (wild type
25 or mutant) of p53 in tumor cells does not always correlate with chemosensitivity. Endogenous p53 status predicts *in vitro* chemosensitivity to the clinically useful agents Adriamycin, Carboplatinum, Cytosan, and Etoposide in only a minority of cancer cell lines
30 (ovarian and some Burkitt's lymphoma cells), but not in others (e.g., leukemia or lung cancer cells). Several recent studies have shown that many clinically useful drugs appear to be more effective in *in vitro* killing of tumor cell lines that contain wild-type p53.
35 Furthermore, HPV16 E6-mediated degradation of p53 in a human lung cancer cell line does not render these cells more resistant to Adriamycin or Etoposide. By contrast,

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human ovarian teratocarcinoma cells become more chemoresistant and display less death by apoptosis when the endogenous p53 protein is degraded by E6 expression. Additionally, mutations in the p53 gene do not correlate
5 with radioresistance in a series of human squamous carcinoma cells. Finally, recent studies have shown decreased patient survival in patients who have aggressive tumors which have lost p53 following chemo- or radiotherapy (Velculescu, et. al., 1996, supra).

10 Certain cytokines interacting with their cognate receptors on the surface of target cells also trigger apoptosis. For example, Fas ligand (FasL) and tumor necrosis factor (TNF) are members of the TNF family of cytokines that trigger apoptosis in target cells
15 through binding and trimerization of their respective cell surface receptors, Fas and TNF receptor 1 (TNFR1). Another TNF family cytokine is TRAIL, the cytotoxic ligand for the recently cloned DR4 receptor (Pan et al., Science 276: 111-113, 1997). Once trimerized, the death
20 domains in the C-termini of the receptors form protein-protein interactions with the C-terminal death domains of intracellular death signal effector molecules, initiating a cascade of signals (involving activation of caspases and endonucleases) that ultimately cause apoptosis.

25 Wild-type p53 has been shown to upregulate production of Fas/APO-1 mRNA and protein, but as yet, no p53-responsive DNA binding site has been identified in the *fas* gene. Thus p53 has not been shown to directly regulate the transcription of a TNFR family member in an
30 apoptosis pathway.

It is clear from the foregoing discussion that the relationship between p53 status and chemo- and radiosensitivity is not sufficiently understood to warrant its use in cancer therapy. As set forth above,
35 p53 status in a tumor does not necessarily predict therapeutic outcome following such treatment. Moreover, gene replacement therapy with wild-type p53 may be

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ineffective, and for some tumors counterproductive, as it is not as yet predictable which tumors would respond positively or negatively to such therapy. For these reasons, there is a need to identify downstream targets of p53 function in apoptotic signaling. Once identified, such p53 targets may be utilized or regulated in apoptosis-based therapeutic approaches to the elimination of tumor cells.

10 SUMMARY OF THE INVENTION

The present invention provides novel nucleic acid molecules that are themselves targets of p53 activity and whose encoded proteins are involved in modulation of cellular proliferation. According to one aspect of the present invention, an isolated nucleic acid molecule is provided that includes an open reading frame encoding a mammalian p53-inducible, apoptosis-mediating protein. The protein comprises an amino-terminal extracellular domain, a transmembrane domain, and a death domain that facilitates protein-protein interactions.

In a preferred embodiment of the invention, the nucleic acid molecule is of human origin, and comprises a sequence substantially the same as SEQ ID NO:1, or at least substantially the same in the death domain-encoding region as SEQ ID NO:1. The invention also comprises a nucleic acid having the sequence of SEQ ID NO:1. The encoded protein, which is referred to herein as "Killer," for reasons described below, preferably comprises a sequence substantially the same as SEQ ID NO:2, or at least substantially the same in the death domain of SEQ ID NO:2. A Killer protein encoded by SEQ ID NO:2 is another preferred embodiment of the invention.

In another aspect of the invention, oligonucleotides that specifically hybridize with selected portions of the nucleic acid molecules of the invention are provided.

In yet another aspect of the invention,

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antibodies immunologically specific for the Killer proteins of the invention, or fragments thereof, are provided.

According to other aspects of the present invention, uses of the nucleic acid molecules of the invention, their encoded proteins, as well as antibodies to those proteins, for diagnostic and therapeutic purposes are provided. Specifically, methods are provided for assessing the induction of "Killer" expression following exposure of tumor cells to chemotherapeutic agents. In accordance with the present invention, Killer expression is assessed in conjunction with p53 status. These methods will facilitate identification of efficacious chemotherapeutic agents as measured by the induction of apoptosis in the targeted tumor cell.

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims. The terms "substantially the same," "percent similarity" and "percent identity (identical)" are defined in detail in the description set forth below.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from

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RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

5 With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may
10 refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

 The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the
15 compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the
20 compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

 With respect to antibodies of the invention, the term "immunologically specific" refers to antibodies
25 that bind to one or more epitopes of a protein of interest (e.g., "Killer"), but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

 With respect to oligonucleotides, the term
30 "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially
35 complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded

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DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

5 The nucleic acids, proteins and antibodies of the present invention are useful as diagnostic and therapeutic agents for the detection and treatment of cancer and other proliferative diseases. They should also find utility as research tools and will facilitate
10 the elucidation of the mechanistic action of the novel genetic and protein interactions involved in the control of chemotherapy induced cell death.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Fig. 1 depicts the nucleic acid sequence of *Killer*, a novel adriamycin-inducible transcript. The figure shows the encoding nucleotide (SEQ ID NO:1) and predicted protein translation (SEQ ID NO:2) of the *Killer* open reading frame. The death domain is enclosed in a
20 box, and the putative transmembrane domain and signal peptide sequences are underlined by double and single lines, respectively. The termination codon is indicated by a star. *Killer* has a perfect Kozak sequence upstream of the initiating methionine 5'-CCGCCATGG-3'.

25 Figs. 2A and 2B show protein sequence homology between *Killer* and related proteins. Amino acid homology between *Killer* and its closest homolog DR4 (SEQ ID NO:3) is depicted in Fig. 2A. In the case of DR4, only the predicted mature protein sequence is shown (Pan et al.,
30 1997, *supra*). Identity and similarity are indicated by a star and a colon, respectively. The pairwise alignment was carried out using the MacVector™ 6.0 Clustal W(1.4) program (Oxford Molecular Group). Fig. 2B indicates the homology in the death domain regions of *Killer* and other
35 TNF receptor family members. A multiple sequence alignment algorithm was carried out using the MacVector™ 6.0 Clustal W(1.4) program (Oxford Molecular Group). A

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predicted death domain consensus is shown below the alignment. The degree of greyness reflects the degree of conservation between different family members. The following amino acids were included within the death domain of each family member: Killer aa 324-393 (part of SEQ ID NO:2); DR4 aa 356-404 (part of SEQ ID NO:3), DR3 aa 346-392 (SEQ ID NO: 4), Fas/apo1 aa 228-277 (SEQ ID NO:5), TNFR-1 aa 330-380 (SEQ ID NO:6), and CAR 1 aa 315-333 (SEQ ID NO:7).

10 Figs. 3A, 3B and 3C show regulation of *Killer* gene expression by wild-type p53. Fig. 3A: Northern analysis of *Killer* expression (upper panels) was carried out using RNA derived from different human cancer cell lines (as indicated) either untreated (-) or 10 hrs after continuous treatment (+) with an apoptosis-inducing concentration 0.3 μ g/ml of doxorubicin (adriamycin, adria) or 500 Rads ionizing radiation. Fig. 3B: Northern analysis shows that *Killer* mRNA expression is increased in H460 lung cancer cells 10 hours after continuous treatment with 0.3 μ g/ml doxorubicin (adriamycin), 5 μ M Etoposide or a dose of 2,000 rads ionizing radiation at time zero. Fig. 3C: Northern analysis showing induction of *Killer* mRNA expression in mutant p53-expressing colon, ovarian or breast cancer cells 12 hours after infection by either Ad-LacZ or Ad-p53. An ethidium stain of the RNA (lower panels) indicates equivalent RNA loading (10 μ g) of the lanes in each experiment, and the p53 status of each cell line is shown below the ethidium stains (Figs 3A, 3C).

30 Fig. 4 is a graph illustrating that expression of the death domain in *E. coli* is toxic. Bacteria were transformed with pGEX-GD vector encoding the death domain of *Killer* in the presence (open squares, open circles) or absence (closed squares, closed circles) of glucose. Induction of death domain expression by IPTG resulted in a dramatic decrease in absorbance which was unaffected by the presence or absence of glucose ruling out a

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catabolite repression effect.

Fig. 5 shows that *Killer* is not induced in p53-dependent G1 arrest. Fig 5A: to induce p53-dependent cell cycle arrest in the absence of DNA damage, GM cells carrying a dexamethasone-inducible wild-type p53 exogenous transgene were incubated in the absence (lane 1) or presence (lanes 2-4) of dexamethasone. After incubation for increasing periods of time (as indicated) total RNA was isolated and Northern blot analysis, using either the human *Killer* (upper panel) or the human p21 (middle panel) cDNA as probes, was performed as described in Materials and Methods. An ethidium bromide stain of the RNA is shown (lower panel) to document equivalent loading of the gel. Fig. 5B: to induce a DNA damage/p53-dependent cell cycle arrest, WI38 cells were exposed to either 2 Gy or 10 Gy g-radiation. Total RNA was harvested at 10 hrs and Northern blotting performed as in A using either the *Killer* (upper panel) or the p21 (middle panel) cDNA as a probe. An ethidium bromide stain of the RNA is shown (lower panel) to document equivalent loading of the gel.

Figs. 6A and 6B show expression of *Killer* in cells undergoing apoptosis following Ad-p53 infection. Fig. 6A: expression of *Killer* is increased in GM cells undergoing apoptosis but not growth arrest. Northern blots of *Killer* mRNA expression in GM cells that were untreated (lane 1), treated with dexamethasone to induce a p53-dependent cell cycle arrest (lane 2), infected by Ad-LacZ as a control (lane 3) or infected by Ad-p53 (lane 4). An ethidium bromide stain is shown (below; 28S and 18S RNA indicated by arrows) to document equivalent loading of the RNA samples. Fig. 6B: p53-dependent upregulation of *Killer* mRNA expression occurs early in SKOV3 cells and continues for the next 48 hrs. Northern analysis of total RNA harvested at increasing time points (as indicated) following infection of SKOV3 cells by Ad-LacZ (lanes 2, 4, 6, 8, 10) or Ad-p53 (lanes 3, 5, 7,

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9, 11) for *Killer* expression.

Figs. 7A and 7B show transcriptional control of TRAIL death receptor expression in p53-dependent apoptosis. The human ovarian carcinoma cell line SKOV3 (Fig. 7A) or the human glioblastoma cell line GM (Fig. 7B) were infected by Ad-LacZ (lanes 1,2) or Ad-p53 (lanes 3,4). Following infection for one hour, cells were incubated either in the absence ("-" as indicated; lanes 1,3) or in the presence ("+" as indicated; lanes 2,4) of 30 ng/ml Actinomycin D for an additional 20 hrs when total cellular RNA was harvested for northern blotting for expression of *Killer*, *p21*, *MDM2*, and *p53* mRNA (as indicated). An ethidium stain of the RNA is shown (lowest panels) to document equivalent loading of the gel.

Fig. 8 is a diagram of the physical map of the coding region of the human *Killer* gene. Exons are shown as black boxes; the number of the exon ("e") together with its size (in kb) are indicated above the boxes. The introns are shown as clear boxes and their respective sizes (in kb) are written within. The double headed arrows below represent the products of PCR reactions, shown together with their respective sizes. The translation initiation codon is depicted with a bent arrow, and the translation stop codon with a blunted arrow. The genomic position of the signal sequence (SS), the transmembrane domain (TM) and the death domain (DD) are indicated above the bar. The positions and the number of the extracellular Cysteine residues (C) are drawn above the bar. The gray boxes represent the position of clones obtained through subcloning P1-Killer, PstI digestion fragments (hybridizing to probes-A and B on Southern blots, shown above the boxes), into pBluescript.

Fig. 9 is the nucleotide sequence of a genomic clone of *Killer*, "P1-Killer" (SEQ ID NO:14). The putative CAT and TATA boxes are indicated, as is the putative start codon.

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DETAILED DESCRIPTION OF THE INVENTION

5 As described above, p53 plays a pivotal and complex role in preventing malignant transformation. In some instances, p53 has been found to activate apoptosis after treatment of cells with chemotherapeutic agents or radiation. However, the relationship between p53 status and sensitivity or resistance to such treatments remains unclear, making it difficult to design or implement therapeutic strategies based on p53. Identification of downstream targets of p53 function in apoptotic signaling would help overcome this difficulty. One such target has now been identified in accordance with the present invention.

 Using a subtractive hybridization technique, the inventors have identified a unique cDNA clone corresponding to a gene whose expression is upregulated in Adriamycin-treated chemosensitive teratocarcinoma cells. The pattern of inducibility of the gene product correlates well with the p53 status of the cell lines, indicating that expression of the gene is regulated by p53. The cloning and analysis of that cDNA is described in detail below.

 From the deduced amino acid sequence of the cDNA, it has been determined that the gene encodes a protein that is a new member of the tumor necrosis factor receptor (TNFR) protein family. This protein is referred to herein as "Killer" or "Killer protein", in view of its presumed role in induction of apoptosis. The genomic DNA or cDNA that encodes the Killer protein is sometimes referred to herein as the "killer" gene or cDNA.

 The TNFR family includes two TNFRs (TNFR1 and TNFR2), the receptor for lymphotoxin- β , nerve growth factor (NGF) receptor (p75), CD40, CD27, CD30, DR3 (death receptor-3), DR4, HVEM (herpes virus early mediator), and

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CAR1 (cytopathic avian leukosis-sarcoma virus receptor. See Beutler, B. & Van Huffer, *Ann. NY Acad. Sci.*, 730:118-133 (1994), S. Nagata, *Cell*, 88:355-365 (1997), G. Pan, et al., *Science*, 276:111-113 (1997). The Killer protein of the invention shares general structural features of the TNFR family in that it is believed to comprise three domains: (1) an extracellular domain at the amino terminus; (2) a transmembrane domain in the central region; and (3) a carboxyl "death" domain. Functionally, Killer is believed to behave in general like a TNFR: the extracellular domain functions as receptor for a cognate extracellular ligand (analogous to TNF for TNFR1); binding of the ligand to the extracellular receptor domain initiates a conformational change through the transmembrane domain to the death domain, which transduces a signal (or cascade of signals) within the cell that ultimately induces apoptosis.

A comparison of Killer with the closely related members of the TNFR (specifically focusing on the death domain) family is set forth in greater detail in Example 1 and shown in Figures 1B and 2. As can be seen, though Killer shares sufficient amino acid sequence similarity with other members of the TNFR family to warrant including it within that family, it is a unique member of that family. The closest homolog is DR4, which shares only about 64% amino acid sequence identity with Killer, even in the most conserved death domain, and about 60% identity comparing all of SEQ ID NO:2, which comprises the death domain, the transmembrane domain and the extracellular domain. Moreover, expression of the Killer gene appears to be regulated by p53, presumably through one or more p53 binding sites. The gene that encodes DR4 has not been reported to be regulated by p53.

Killer also appears to be unique in that it is regulated by DNA damage. Results obtained by the present inventor demonstrate that, whereas Killer mRNA expression is induced by DNA damage in wild-type p53 containing

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cells, so such universal phenomenon was observed for either DR4 or the decoy receptor, TRID.

Loss of Killer's apoptotic function has also been linked to a human cancer. Transfections of head and neck squamous cell carcinoma, colon and ovarian carcinoma cell lines revealed loss of growth-suppressive function associated with a *Killer* truncation mutant derived from head and neck tumor.

Example 2 describes further important characteristics of *Killer*. In that Example it is shown that, unlike the *p53*-target CDK-inhibitor *p21^{WAF1/CIP1}*, *Killer* is only induced in cells undergoing *p53*-dependent apoptosis and not cell cycle arrest. Inhibition of transcription by Actinomycin D blocks both *Killer* and *p21* gene induction in cells undergoing *p53*-dependent apoptosis, indicating that *p53* regulation of *Killer* is transcriptional.

The DNA of the invention encodes a *Killer* protein. The gene, its corresponding cDNA and encoded protein have been designated *Killer* due to its involvement in the apoptotic pathway. The gene is turned on early in the dying process in chemotherapy treated chemosensitive, but not chemoresistant ovarian cancer cells. Apoptosis is a type of cell death that is thought to be under direct genetic control. During apoptosis, cells lose their cell junctions and microvilli. The cytoplasm condenses and nuclear chromatin marginates into a number of discrete masses. While the nucleus fragments, the cytoplasm contracts and mitochondria and ribosomes become densely compacted. After dilation of the endoplasmic reticulum and its fusion with the plasma membrane, the cell breaks up into several membrane bound vesicles, also known as apoptotic bodies, which are usually phagocytosed by adjacent cells.

The death domain of *Killer* is highly homologous to such domains in other known death receptors that cause apoptosis in response to a variety of other signals. In

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accordance with the present invention, it has also been shown that the expression of the death domain in *E. coli* is toxic.

Figure 1 sets forth the nucleotide sequence
5 (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID
NO:2) of a cDNA of human origin encoding Killer protein
of the invention. SEQ ID NO:1 comprises the complete
coding sequence of Killer. The protein contains a
transmembrane domain, a C-terminal death domain, and an
10 N-terminal extracellular domain. This sequence
information indicates that Killer may be classified as a
new member of the TNFR family, as discussed above.

Although the human Killer-encoding gene is
described and exemplified herein, the present invention
15 also encompasses nucleic acid sequences and proteins from
other species that are functionally and structurally
homologous to be used interchangeably with Killer-
encoding nucleic acids and proteins for the diagnostic,
therapeutic, and research purposes described below.
20 Because of the high degree of conservation of genes
encoding specific signal transducers and related
oncogenes, it will be appreciated by those skilled in the
art that, even if the interspecies Killer similarity is
low, Killer-encoding nucleic acids and Killer proteins
25 from a variety of mammalian species should possess a
sufficient degree of homology with Killer so as to be
interchangeably useful with Killer in such diagnostic and
therapeutic applications. Accordingly, the present
invention is drawn to mammalian Killer-encoding nucleic
30 acids and Killer proteins, preferably to Killer of
primate origin, and most preferably to Killer of human
origin. Accordingly, when terms such as "Killer" or
"Killer-encoding nucleic acid" are used herein, they are
intended to encompass mammalian Killer-encoding nucleic
35 acids and Killer proteins falling within the confines of
homology set forth below, of which human Killer is an
exemplary member.

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Allelic variants and natural mutants of SEQ ID NO:1 are likely to exist within the human genome and within the genomes of other mammalian species. Because such variants are expected to possess certain differences
5 in nucleotide and amino acid sequence, this invention provides an isolated nucleic acid molecule and an isolated Killer protein having at least about 50-60% (preferably 60-80%, most preferably over 80%) sequence homology in the coding region with the nucleotide
10 sequence set forth as SEQ ID NO:1, and the amino acid sequence of SEQ ID NO:2. Such nucleic acid sequences will also specifically hybridize with SEQ ID NO:1 or its complement.

Because of the natural sequence variation
15 likely to exist among Killer proteins and nucleic acids encoding them, one skilled in the art would expect to find up to about 40-50% sequence variation, while still maintaining the unique properties of the Killer protein of the present invention. Such an expectation is due in
20 part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the protein. Accordingly, such variants are considered substantially the same as one another and are
25 included within the scope of the present invention.

For purposes of this invention, the term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variations that are conservative or neutral. With particular reference to
30 nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative
35 substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative

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substitutions and/or variations in regions of the polypeptide. The terms "percent identity" and "percent similarity" are also used herein in comparisons among amino acid sequences. These terms are intended to be
5 defined as they are in the UWGCG sequence analysis program (Devereaux et al., Nucl. Acids Res. 12: 387-397, 1984), available from the University of Wisconsin.

The following description sets forth the general procedures involved in practicing the present
10 invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring
15 Harbor Laboratory (1989) (hereinafter "Sambrook et al.") are used.

I. Preparation of Killer-Encoding Nucleic Acid Molecules, Killer Proteins and Antibodies
20 **Immunologically Specific for those Proteins**

A. Nucleic Acid Molecules

Nucleic acid molecules encoding the Killer
25 proteins of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence
30 information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite
35 method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA

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molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a 4.44 kb double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

Nucleic acid sequences encoding Killer may be isolated from appropriate biological sources using methods known in the art. In a preferred embodiment, a cDNA clone is isolated by subtractive hybridization of a cDNA library of human origin. In an alternative embodiment, human genomic clones encoding Killer may be isolated. In fact, genomic clones of Killer have been isolated (an exemplary clone, P1-Killer, is shown in Fig. 9), and a complete physical map of the Killer gene, located on human chromosome 8p21, has been constructed (Figure 8). Alternatively, Killer cDNA or genomic clones from other mammalian species may be obtained.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with a selected region of SEQ ID NO:1 (e.g. the segment encoding the transmembrane domain or the death domain) may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six

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hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65° in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$$

As an illustration of the above formula, using $[N^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such sequences would be considered substantially complementary to the probe.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell. Optionally, the vectors may contain inducible promoter sequences, such as metallothionine, tetracycline or dexamethasone responsive promoters. Such vectors enable the investigator to regulate the expression of killer protein in the transformed tumor cell while assessing induction of apoptosis.

Killer-encoding nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or

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antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the cDNA having SEQ ID NO:1. Such
5 oligonucleotides are useful as probes for detecting *Killer* genes in test samples of potentially malignant cells or tissues, e.g. by PCR amplification, or for the isolation of homologous regulators of apoptosis.

In addition, 5' and 3' regulatory sequences of
10 *Killer*, available on the genomic clone discussed above, can be used to advantage. For instance, the promoter sequence can be cloned upstream of a reporter gene, so that drug screening can be performed to isolate compounds that induce *Killer's* expression. Reporters may include
15 LacZ, Luciferase, GFP, Hygromycin, or other negative or positive selection markers.

B. Proteins

A *Killer* protein of the present invention may
20 be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by immunoaffinity purification. However, this is not a preferred method due to the low amount of protein likely
25 to be present in a given cell type at any time.

The availability of nucleic acids molecules encoding *Killer* enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate
30 *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g.,
35 from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred

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embodiment, larger quantities of Killer may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the cDNA having SEQ ID NO:1 may be inserted into a
5 plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell. Such vectors provide the regulatory elements necessary for expression of the DNA in the given host cell, positioned in such a manner as to
10 permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The Killer protein produced by gene expression
15 in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted
20 from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind
25 specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

The Killer proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, such proteins may
30 be subjected to amino acid sequence analysis, according to known methods.

The present invention also provides antibodies which bind immunospecifically to Killer proteins may be prepared according to standard methods. In a preferred
35 embodiment, monoclonal antibodies are prepared, which react immunospecifically with various epitopes of Killer. Monoclonal antibodies may be prepared according to

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general methods of Köhler and Milstein, following standard protocols. Polyclonal or monoclonal antibodies that immunospecifically interact with Killer can be utilized for identifying and purifying such proteins.

- 5 For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.
- 10 Other uses of anti-Killer antibodies are described below.

15 II. Uses of Killer-Encoding Nucleic Acids, Killer Proteins and Antibodies Thereto

- Cellular signaling molecules, including proteins involved in apoptosis pathways, have received a great deal of attention as potential mediators of selective killing of tumor cells for the elimination of cancer from the body. As a signaling molecule involved in p53-mediated apoptosis, Killer and related proteins from other mammalian species will be particularly useful as diagnostic and therapeutic agents. Such molecules will also provide valuable research tools.

A. Killer-Encoding Nucleic Acids

- Killer-encoding nucleic acids may be used for a variety of purposes in accordance with the present invention. Killer-encoding DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of genes encoding Killer, as well as to identify alterations of killer, resulting in loss of function, as they arise in various cancers. In this regard, it should be noted that the diagnostic value of detecting *Killer* expression, even at the mRNA level, is significant. *Killer* DNA may be hypermethylated or simply not activated after exposure to chemotherapy in tumors that go on to become treatment-resistant. Such

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information may be useful in designing chemotherapy for particular patients. Methods in which Killer-encoding nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The Killer-encoding nucleic acids of the invention may also be utilized as probes to identify related genes either from humans or from other species. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, Killer-encoding nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to Killer, thereby enabling further characterization of the signaling cascade involved in the control of apoptosis in different cell types. Additionally, they may be used to identify genes encoding proteins that interact with Killer (e.g., by the "interaction trap" technique), which should further accelerate elucidation of these cellular signaling mechanisms (Golemis, et al., Interaction trap/two-hybrid systems to identify interacting proteins, Unit 20.1.1-20.1.28 in Current Protocols in Molecular Biology, eds. F.M. Ausubel, et al. John Wiley & Sons, NY (1996). Yeast two-hybrid screening may be used to identify the extracellular ligand(s) for Killer, and the proteins that interact with the cytoplasmic portions of Killer, including death domain-containing protein partners. Killer may function to promote apoptosis through a pathway analogous to the Fas and TNFR1 systems. The Fas apoptosis pathway is activated by the binding of Fas ligand (FasL) to Fas, and TNF-induced apoptosis begins by ligation of TNF to TNFR1. The use of interaction trap/two-hybrid assays will permit the identification of Killer-specific, extracellular ligands

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which trigger the Killer apoptosis cascade. Further, these assays will be used to identify the intracellular proteins that interact with Killer to potentiate the signal within the cell.

5 Nucleic acid molecules, or fragments thereof, encoding Killer may also be utilized to control the expression of Killer, thereby regulating the amount of protein available to participate in apoptosis signaling pathways. Alterations in the physiological amount of
10 Killer protein may act synergistically with chemotherapeutic agents used to treat cancer.

 In one embodiment, the nucleic acid molecules of the invention may be used to decrease production of Killer protein in a population of cells. In this
15 embodiment, Killer proteins would be made unavailable for the transduction of specific apoptosis signals, providing a model system for research purposes. In this embodiment, antisense oligonucleotides are employed which are targeted to specific regions of Killer-encoding genes
20 that are critical for gene expression. The use of antisense oligonucleotides to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, such antisense oligonucleotides are modified in various ways to increase their stability and
25 membrane permeability, so as to maximize their effective delivery to target cells *in vitro* and *in vivo*. Such modifications include the preparation of phosphorothioate or methylphosphonate derivatives, among many others, according to procedures known in the art. Antisense
30 molecules may also be introduced on conventional DNA vectors used for gene therapy. Once in the appropriate target cells, the antisense molecule is produced by expression of a recombinant DNA segment under control of appropriate promoters and other regulatory sequences.

35 In another embodiment designed to decrease Killer expression or activity, inhibitors of such expression or activity are identified and utilized. Such

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inhibitors would be useful not only for laboratory studies, but also therapeutically, as agents that protect normal tissue against the cytotoxic effects of chemotherapy. A similar approach is being taken, for example, using expression of the MDR1 glycoprotein in bone marrow cells. The MDR1 glycoprotein has been found to be overexpressed in tumor cells that have become resistant to chemotherapeutic agents. Inhibitors of MDR1 are useful to counteract this effect. Similarly, if Killer mutates in any tumors and such mutant Killer interferes with cell death pathways, then inhibitors of Killer would be of direct therapeutic value in such cases.

In another embodiment, overexpression of Killer is induced in a target population of cells to generate an excess of Killer molecules. This excess production of Killer may be monitored with immunofluorescence or any other standard technique known in the art. Further, excess production of Killer protein will trigger apoptosis and facilitate the isolation and characterization of the intracellular death domain-containing proteins that interact with Killer and potentiate the apoptosis signal.

Another embodiment of the invention is excess production of the Killer death domain in target cells to assess the effect of the death domain on apoptosis signaling and chemosensitivity.

Another use of the nucleic acids of the invention is in gene replacement therapy for expression of Killer in instances of null or mutant p53 status in a particular target tumor, such that apoptosis may be triggered in tumor cells that will not die in response to chemotherapy. Gene therapy to provide a functional Killer to cells lacking the functional protein is also contemplated. This sort of gene therapy should prove particularly useful in cancers (such as the head and neck cancer described above) specifically associated with a

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disrupted *Killer* gene.

As described above, *Killer*-encoding nucleic acids are also used to advantage to produce large quantities of substantially pure *Killer* protein, or
5 selected portions thereof. In a preferred embodiment, the C-terminal death domain of *Killer* (see Figure 1B) is produced by expression of a nucleic acid encoding the domain. The full-length protein or selected domain is thereafter used for various research, diagnostic and
10 therapeutic purposes, as described below.

B. Killer Protein and Antibodies

Purified *Killer*, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which
15 also may serve as sensitive detection reagents for the presence and accumulation of *Killer* (or complexes containing *Killer*) in cultured cells or tissues from living patients (the term "patients" refers to both humans and animals). Recombinant techniques enable
20 expression of fusion proteins containing part or all of the *Killer* protein. The full length protein or fragments of the protein may be used to advantage to generate an array of monoclonal antibodies specific for various epitopes of the protein, thereby providing even greater
25 sensitivity for detection of the protein in cells or tissue.

Polyclonal or monoclonal antibodies immunologically specific for *Killer* may be used in a variety of assays designed to detect and quantitate the
30 protein, which may serve as a prognostic indicator for malignant disease. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization in *Killer* in cultured cells or biopsy tissue; and (3) immunoblot analysis (e.g., dot
35 blot, Western blot) of extracts from various cells and tissues. Additionally, as described above, anti-*Killer* antibodies can be used for purification of *Killer* (e.g.,

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affinity column purification, immunoprecipitation).

Anti-Killer antibodies may also be utilized as therapeutic agents to block the normal functionality of Killer in a target cell population. Thus, similar to the antisense oligonucleotides described above, anti-Killer antibodies may be delivered to a target cell population by methods known in the art (i.e. through various lipophilic carriers that enable delivery of the compound of interest to the target cell cytoplasm) where the antibodies may interact with intrinsic Killer protein to render it nonfunctional.

Alternatively, the possibility exists that anti-Killer antibodies may in fact activate Killer's receptor and have a therapeutic value in inducing apoptosis. Precedent exists for such a function, for instance, with anti-Fas antibodies. An anti-Killer antibody use for such a purpose may prove superior to the true ligand, and it may be deliverable intravenously, depending on its specificity for cancer cells, and absence of toxicity. The antibody also may be injected directly into a tumor.

From the foregoing discussion, it can be seen that Killer-encoding nucleic acids and antibodies to Killer proteins of the invention can be used to detect gene expression and protein accumulation for purposes of assessing the genetic and protein interactions involved in the regulation of apoptosis in both wild-type p53 positive and negative cells.

Aberrant signal transduction in cells is often correlated with cellular transformation and cancer of various tissue types. It is expected that these tools will be particularly useful for diagnosis and prognosis of human neoplastic disease as described above. Potentially of greater significance, however, is the utility of Killer-encoding nucleic acids, proteins and antibodies as agents to assess and alter the chemosensitivity of a targeted tumor cell. In any of

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their therapeutic uses, the nucleic acids, proteins and antibodies of the invention can be used alone, or as adjuncts to other therapeutic agents.

Although the compositions of the invention have
5 been described with respect to human diagnostics and therapeutics, it will be apparent to one skilled in the art that these tools will also be useful in animal and cultured cell experimentation with respect to various malignancies and/or other conditions manifested by
10 altered patterns of apoptosis or lack of apoptosis in response to DNA damaging agents or ionizing radiation. They can also be used to generate animal model systems, e.g. "knockout" animals that do not express the gene, as a model of cancer susceptibility. Such animals are
15 useful as models for human disease and treatment thereof, inasmuch as they may exhibit phenotypes that may mimic a developmental abnormality and may have a predisposition to cancer, especially following exposure to carcinogens. In connection with this embodiment, it is preferable to
20 clone a mouse homolog of *Killer*, according to standard methods as described above.

The following Examples are provided to describe the invention in further detail. The examples are
25 intended to illustrate and not to limit the invention.

EXAMPLE 1

30 Isolation and Characterization of a Nucleic Acid Molecule Encoding Human Killer Protein

In this Example the cloning of a cDNA molecule encoding human Killer is described. To identify genes transcriptionally regulated by wild-type p53 in response
35 to DNA damage, a cDNA library enriched for the presence of such genes was constructed and screened by a subtractive hybridization technique. The cell line used as a source for the cDNA library was PA-1, a human, wild-type p53-positive, ovarian teratocarcinoma. p53 status

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correlates with chemosensitivity in these cells (Wu & El-Deiry, Nature Med. 2: 255-256, 1996).

Materials and Methods

5 **Cell lines and culture conditions.** The human
leukemia cell line ML-1, a gift from Michael B. Kastan
(Johns Hopkins University, Baltimore, MD), the human lung
cancer cell line H460, a gift from Stephen B. Baylin
(Johns Hopkins University, Baltimore, MD) and the human
10 colon carcinoma cell line HCT-116, a gift from Dr. Bert
Vogelstein (Johns Hopkins University, Baltimore, MD) were
maintained in RPMI 1640 supplemented with 10% fetal
bovine serum (FBS), 1% penicillin and streptomycin (Wu
et al., Clin. Cancer Res. 2: 623-633, 1996; Prabhu et
15 al., Clin. Cancer Res. 2: 1221-1229, 1996). The human
ovarian cancer cell lines PA-1 and SKOV3, and the human
breast cancer line SKBr3 were obtained from the American
Type Culture Collection (Rockville, MD) and maintained as
previously described (Wu et al., 1996, *Supra*;
20 Blagosklonny & El Deiry, Int. J. Cancer 67: 386-397,
1996). The human colon carcinoma cell line SW480 was
obtained from the University of Pennsylvania Cell Center
and maintained as previously described (Fuchs et al.,
Cancer Res. 57: 2550-2554, 1997). HPV-E6 expressing
25 clones of HCT-116, PA-1, and H460 were transfected and
maintained as previously described (Wu & El Deiry, Nature
Med. 2: 255-256, 1996; Wu et al., 1996, *supra*; Prabhu et
al., 1996, *supra*). Cells were grown in the presence of
0.3 µg/ml adriamycin for 10 hrs followed by isolation of
30 total RNA as previously described (El Deiry et al., Cell
75: 817-825, 1993). H460-neo and H460-E6 cells were
irradiated at 500 rads and grown for 10 hrs followed by
isolation of total RNA as previously described (Fuchs et
al., 1997, *supra*; El Deiry et al., 1993, *supra*).
35 Replication-deficient adenovirus recombinants expressing
β-galactosidase (Ad-LacZ) or wild-type p53 (Ad-p53) were
gifts from Dr. B. Vogelstein (Johns Hopkins University),

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and were prepared, titered, and used as previously described (Fuchs et al., 1997, *supra*; Blagosklonny & El Deiry, 1996, *supra*; El Deiry et al., 1993, *supra*). Cell lines were infected at a multiplicity of infection (MOI) of 50, and total protein and RNA were harvested 20 hrs later. The infectivity of the cell lines was determined by X-gal staining, as previously described (Blagosklonny & El Deiry, 1996, *supra*).

Subtractive hybridization screening. PA1-neo ovarian teratocarcinoma cells (Wu & El Deiry, 1996, *supra*) were incubated for 10 hrs in the presence of 0.3 μ g/ml adriamycin. A cDNA library was constructed using poly(A+) RNA prepared from the adriamycin-treated cells. A total of 50,000 plaques were differentially screened by hybridization to the following: (1) a cDNA probe prepared from equal portions of RNA isolated from untreated PA1-neo cells and PA1-E6 cells, and (2) a subtracted probe prepared by using 500 ng cDNA from adriamycin-treated PA1-neo cells as target and a total of 20 μ g driver poly (A+) RNA (10 μ g from untreated PA1-neo cells and 10 μ g from adriamycin-treated PA1-E6 cells). The cDNA library and probes were prepared using previously described methods (El Deiry et al., 1993).

The initial isolate of the *Killer* cDNA was 0.6 kb of the extreme 3'-untranslated region and this hybridized to a 4.4 kb mRNA species. Hybridization of this probe to the original oligo dT-primed adriamycin-treated PA1 cDNA library yielded a 2.0 kb fragment containing 3'-untranslated *Killer* sequence with no homology in the Genbank database. We performed 5'-RACE PCR to obtain the 5'-coding sequence of *Killer* using the Marathon™ cDNA Amplification Kit (Clontech).

Northern Blot Analyses. Northern blot analyses were performed using either 10 μ g of total cellular RNA (Figs. 1A,3) or 2 μ g poly (A+) RNA (Multiple Tissue Northern (MTN™) Blot from Clontech) as previously described. The PA1-neo/E6 northern blot was probed with

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a 600 bp 3' untranslated region of the *Killer* cDNA. All other northern blots were probed with a 1.14 kb fragment from the C-terminal coding region of the *Killer* cDNA. In addition, a 2.0 kb human β -actin probe (Clontech) was used to probe the MTN[™] blot. p21 mRNA expression was detected as previously described (Fuchs et al., 1996, *supra*; El Deiry et al., 1993, *supra*).

Plasmids. The full-length open reading frame of the *Killer* cDNA was amplified by RT-PCR from adriamycin-treated PA1 cellular RNA using 5'-CCCAAGCTTCCGCCATGGAACAACGGGGAC-3' (SEQ ID NO:8) and 5'-CGCGGATCCTTAGGACATGGCAGAGTCTGC-3' (SEQ ID NO:9) and the following PCR conditions: 35 cycles of denaturation at 95°C for 30 seconds followed by annealing at 68°C for 90 seconds and extension at 72°C for 60 seconds. This PCR product was subcloned into the pCRII vector using the TA-cloning kit (Invitrogen) and following restriction by BamHI and HindIII the cDNA was cloned into the mammalian expression vector pCEP4 (Invitrogen). The cytoplasmic domain was obtained by PCR amplification (30 cycles: 95°C for 30 sec, 65°C for 1 min, 72°C for 1 minute) using *Killer* cDNA as template and the following primers: 5'-CGCGGATCCGTCCTCAATCTTCTGCTTGG-3' (SEQ ID NO:10) and 5'-CCCAAGCTTCCACCATGGTTGCAGCCGTAGTCTTG-3' (SEQ ID NO:11). The death domain was amplified using the primers 5'-CCCAAGCTTCCACCATGGACTCCTGGGAGCCGC-3' (SEQ ID NO:12) and 5'-CGCGGATCCGTCCTCAATCTTCTGCTTGG-3' (SEQ ID NO:13) and the same PCR conditions as the cytoplasmic domain. The PCR products were restricted with BamHI and HindIII and subcloned into pCEP4. For *in-situ* RNA hybridization, nucleotides 106 to 726 of *Killer* (Figure 1) by PCR amplification and TA-cloning into the pCR[™]II (Invitrogen) vector in either sense or antisense orientation.

Transfections and apoptosis assays.

Transfection of SW480 cells was carried out as previously described (El Deiry et al., 1993, *supra*). TUNEL assays

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were performed using SW480 cells transfected in 8-well chamber slides. After 36 hrs, TUNEL-positive cells were detected using the Apoptag Plus *In-situ* apoptosis detection kit-Fluorescein (ONCOR). Transfection efficiency in these experiments was 20%.

Chromosomal localization. Fluorescence *in-situ* hybridization (FISH) was performed by standard techniques as previously described (Krantz et al., Am. J. Med. Genet. 70: 80-86, 1997). Three P1 clones (Genome Systems) containing the *Killer* gene were labeled by nick translation with biotin-11-dUTP and hybridized to metaphase spreads from a normal individual. Chromosomes were counter-stained with 4,6-diamino-2-phenyl-indole (DAPI). Analysis was carried out on a Vysis imaging system and after FISH, DAPI staining was computer converted to a G-banding image for chromosome and band identification.

In-situ mRNA expression. *In-situ* hybridization of frozen sections was performed using a modified version of the digoxigenin labeled method as described (Panoskaltsis-Mortari & Bucy, BioTech 18: 300-307, 1995). In brief, colonic tissue was snap frozen in OCT and stored at -80°C. Five μ m sections were cut and placed on Probe-On slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were thawed at room temperature for 1-2 minutes and fixed in 3% paraformaldehyde for 1 minute at room temperature. After one wash with 2 x SSC, the slides were incubated for 8 minutes in 0.2 M HCl and rinsed with 0.1 M triethanolamine, pH 8.0. The slides were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 15 minutes followed by a rinse with 2 x SSC. Pre-hybridization was performed at 50°C for 30 minutes in a solution containing 50% formamide, 4 x SSC, 1 x Denhardt's, 10% dextran sulfate, 500 μ g/ml of heat denatured salmon sperm DNA, and 250 μ g/ml of yeast tRNA. The slides were then hybridized in pre-hybridization solution containing heat-denatured

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(80°C) RNA probe at 50°C overnight. Following hybridization, the slides were rinsed in 2 x SSC for 5 minutes and STE (500 mM NaCl, 20 mM tris-HCl, pH 7.5, 1 mM EDTA) for 1 minute. After incubation in RNase A (40
5 µg/ml in STE) for 30 minutes at 37°C, the slides were washed with 2 x SSC containing 50% formamide for 5 minutes at 50°C followed by three washes with 1 x SSC and 0.5 x SSC, each for 5 minutes. Immunological detection of digoxigenin was carried out using a
10 sheep-anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN). Digoxigenin labeled RNA probes for Killer (both sense and anti-sense) were transcribed from the T7 promoter of pCR^{II} (Invitrogen), after linearization by digestion with HindIII, using the
15 Riboprobe Gemini System 2 kit (Promega) and the Dig/Genius⁴ RNA Labeling Kit (SP6/T7; Boehringer Mannheim).

Targeting p53 for destruction. To generate a tumor cell line in which p53 function was lost, PA-1
20 cells were transfected with a human papilloma virus (HPV) 16 E6 expression plasmid that expresses E6 protein constitutively driven by the cytomegalovirus (CMV) immediate early promoter. PA-1 cells were transfected with either pCMV-neo-bam (referred to herein as either
25 PA-1/neo or "wild-type") or pCMV16E6 (referred to herein as either PA-1/E6 or "degraded" to reflect the function of the E6 protein in degrading p53), as described previously (El-Deiry et al., 1993, *supra*). Individual clones were isolated following selection in the presence
30 of 500 µg/ml G418 (Life Technologies, Inc.), as described by Baker et al., Science 249: 912-915 (1990).

Expression of the Death Domain of Killer in *E. coli*. *E. coli* cells were transformed with a pGEX vector expressing the death domain of the killer protein.
35 Bacterial cells were seeded at low density and grown up overnight in L broth + ampicillin. SDS-PAGE was performed which confirmed that the protein was expressed

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following induction with IPTG. The toxicity assay was performed in the presence and absence of glucose to rule out catabolite repression effects. Toxicity of the death domain was assessed by measuring absorbance at 520 nM in a spectrophotometer over a 5 hour time course.

Results

The p53 tumor suppressor is the most commonly mutated gene in human cancer, and inherited mutations lead to the cancer-prone Li-Fraumeni syndrome. There is evidence that p53 is a key determinant of the sensitivity of mammalian cells to the cytotoxic effects of chemo- and radio-therapy. Biochemical studies have demonstrated that the majority of tumor-derived mutants of p53 have lost the ability to activate gene expression, suggesting that transcriptional activation by p53 may be critical for its role in the control of cell proliferation. Stabilization of p53 protein following virus infection, nucleotide depletion, hypoxia or DNA damage leads to cell cycle arrest and/or apoptosis. Cell cycle arrest is believed to be mediated by transcriptional activation of p21^{WAF1/CIP1}, a universal inhibitor of cyclin-dependent kinases and PCNA-dependent processive DNA replication. p53 upregulates expression of several genes involved in apoptosis, including bax and Fas/APO1. However, the fact that neither bax- nor Fas/APO1-null cells are deficient in DNA damage-induced (p53-dependent) apoptosis suggests that other mechanisms likely contribute to this process.

We previously found that targeted degradation of the p53 protein by HPV16 E6 overexpression in PA1 ovarian teratocarcinoma cells led to inhibition of adriamycin- induced apoptosis and chemoresistance. In order to explore the pathway of cell death in the chemosensitive PA1 cells, we carried out subtractive hybridization screening of a library enriched for adriamycin-induced transcripts (see Methods). Of the

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50,000 phage clones representing PA1 mRNA's expressed at 10 hrs following adriamycin treatment, 100 appeared to hybridize more strongly to a subtracted vs. control probe in primary screening. In secondary screening, 30 of the initially chosen 100 plaques reproducibly displayed differential subtractive hybridization and were then used individually as probes of Northern blots carrying RNA from untreated vs. adriamycin-treated PA1 cells. *p21^{WAF1/CIP1}* was one of six clones whose expression was increased following adriamycin treatment, and its presence provided an indication that the subtraction had yielded at least one expected target. Another known gene that was isolated was *Cathepsin D*, a gene encoding a protease that was also recently isolated as an antisense suppressor of cytokine-induced apoptosis. Of the other four (novel) cDNA's, one appeared to be strongly induced by adriamycin (doxorubicin) and, thus, was further characterized. Database analysis revealed that this novel gene (Figure 1) is a member of the TNF-receptor family. Based on the presence of a cytoplasmic death domain (Figure 1, induction of its expression by cytotoxic chemotherapy (Figure 3) and induction of apoptosis by its overexpression, the novel gene was named *Killer*.

The *Killer* gene encodes a 411 amino acid polypeptide (Figure 1) of predicted molecular weight of 45 kD. The protein contains a cytoplasmic C-terminal death domain, a transmembrane domain, and a cysteine-rich extracellular N-terminal domain characteristic of the TNF-receptor family. At the protein level, *Killer*'s closest homolog is DR4 with 55% identity and an additional 9% similarity over a 454 amino acid alignment. See Figure 2A. Figure 2b shows homology between the death domain of *Killer* and other TNF-receptor family members. This alignment revealed that within the death domain, *Killer* shares 64% identity with DR4, 28% with DR3, 19% with *Fas/APO1*, 29% with *TNFR-1*, and 31% with *CAR1* (Fig. 2B).

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We examined the tissue expression pattern of the *Killer* gene by Northern (RNA) blot analysis. Tissues screened were spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. A single transcript of 4.4 kb hybridized to the *Killer* probe and its expression appeared higher in tissues with rapid turnover as compared with nondividing tissues.

Because *Killer* was isolated as an adriamycin-induced transcript, and because adriamycin is a potent inducer of p53 and p53-dependent growth suppression, we explored the possibility that *Killer* may be a p53-regulated gene. We examined *Killer* mRNA expression in a series of 11 human cancer cell lines of known p53 status. Figures 3A and 3B show that *Killer* expression was induced following adriamycin exposure only if wild-type p53 was present but not in cells where it was mutated, degraded, or not expressed. These results strongly correlated p53-status with induction of *Killer* gene expression following exposure of (11/11) human cell lines to the DNA damaging Topoisomerase II inhibitor adriamycin. We further found that *Killer* expression could be induced by other DNA damaging agents such as ionizing radiation or etoposide (Fig. 3B for example). In order to more directly test the hypothesis that *Killer* is a p53-regulated gene, we infected human cancer cell lines lacking wt p53 by using a wt p53-expressing adenovirus. Fig. 3C shows that in each case tested, *Killer* gene expression was increased by wt p53 overexpression in a manner similar to p21 (not shown). These results suggested that *Killer* is a gene whose expression is increased by the p53 tumor suppressor, even in the absence of DNA damage, raising the possibility that *Killer* may be a mediator in p53-dependent apoptosis. This possibility is further supported by the fact that *Killer* expression is elevated early (increased at 10-12

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hrs after exposure to adriamycin, gamma-radiation or Ad-p53) in cells that are undergoing apoptosis over a 24-48 hr time course.

The identification of *Killer* as a novel p53-
5 and DNA damage-inducible death receptor suggested the possibility that *Killer* inactivation may contribute to tumor development and to resistance to apoptosis *in vivo*. In order to further explore this possibility, we determined the chromosomal localization of the *Killer*
10 gene by *in-situ* hybridization using as probes three P1-clones containing the gene. Ten metaphases were examined for each P1 clone, and signal was detected on both chromosome 8's in all cells studied. The *in-situ* hybridization indicated that it localizes to chromosome
15 8p21, a hotspot for translocations in a number of different tumor types. Because recent experiments have demonstrated that the *Type II TGF- β receptor* and the *bax* genes may be mutated in colon cancers with microsatellite instability, we sequenced the death domain of *Killer* from
20 normal DNA and colon cancer cell lines including 8 with microsatellite instability. We found no alteration involving a GAGAGAGA dinucleotide repeat located within the death domain of *Killer* (data not shown).

Because rapidly dividing tissues such as the
25 gut express increased levels of *Killer*, we investigated its *in-situ* mRNA expression to determine if it correlates with apoptosis in human colon. We found that *Killer* is expressed in the proliferating/stem cell compartment of colonic crypt epithelia, a location which correlates with
30 both spontaneous- and irradiation-induced apoptosis. We hypothesized that *Killer* expression may be downregulated in colonic tumor progression as a potential mechanism of inactivation in cancer. We found that *Killer* mRNA
expression is low or undetectable in a colonic adenoma
35 and two colon carcinomas with p53 mutation. In addition, the Northern blot results in Fig. 3A show that the basal expression (in the absence of adriamycin exposure) of

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Killer is lower in mutant *p53*-expressing SW480 cells and the *p53*-degrading HCT116-E6 cells as compared to wild-type *p53*-expressing HCT116-neo cells. It may be that loss of *Killer* expression in colon cancer may be due to *p53* mutation. In this regard, the extreme 5'-end of the *Killer* gene (exon 1) has been previously isolated as a methylated CpG island (Genbank accession # Z66083). DNA methyltransferase expression level has been found to be increased in colonic polyps, at a stage of tumorigenesis that precedes the development of *p53* mutation. It is possible that *Killer* gene expression may be regulated by DNA methylation, an epigenetic phenomenon that has been found to inactivate a number of tumor suppressor genes in cancer.

We investigated the role of *Killer* in apoptosis by transfecting human colon cancer cells with vectors that express the full-length *Killer* cDNA, its cytoplasmic domain, or the death domain. Results showed that overexpression of *Killer*'s death domain is sufficient to induce apoptosis in transfected cells. The apoptosis observed with the *Killer* expression vectors was similar in magnitude to *p53*-induced apoptosis in these cells (35% vs. 32% TUNEL-positive cells).

The death domain of *Killer* has been cloned into a pGEX vector. Following transformation of *E. coli* cells, and induction of protein expression with IPTG, growth of bacteria was assessed over a 5 hour period. The data in Figure 4 show that expression of the death domain is also toxic to bacteria. This toxicity was not a result of catabolite repression as shown by the +/- glucose controls.

We also investigated the effect of *Killer* on growth of colon and liver cancer cells. SW480 human colon cancer cells were transfected with either pCEP4 vector or pCEP4-killer, according to protocols described above. Transfected cells were selected with 0.25 mg/ml Hygromycin for two weeks, and stained with Coomassie

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Blue. The results of this experiment clearly demonstrated that killer is a potent inhibitor of human colon cancer cell growth: cells transfected with the pCEP4-killer construct were unable to grow, while cells
5 transfected with vector alone were able to grow. Similar results were obtained with HepG2 hepatoblastoma cells.

In summary, we have isolated a novel cell death receptor (*Killer*) whose expression is induced by DNA damaging agents in a p53-dependent manner. *Killer* gene
10 expression is also inducible by wt p53 overexpression in the absence of DNA damage. Overexpression of *Killer* leads to cell death of cancer cells, and a number of human cancers have decreased expression of the *Killer* gene. Members of the TNF receptor family signal
15 apoptosis through adaptor molecules that directly activate the caspase cascade. The identification of *Killer* provides a potential mechanism by which DNA damaging agents can lead to p53-dependent apoptosis.

20

EXAMPLE 2

Involvement of Killer in p53-Dependent and -Independent Apoptosis but Not Growth Arrest

Using the subtractive hybridization approach
25 described in Example 1 to isolate adriamycin-inducible transcripts from chemosensitive cells, we cloned the death receptor *Killer* as a DNA damage-inducible p53-regulated gene on chromosome 8p21. Overexpression of *Killer* induces apoptosis of human cancer cells and
30 eliminates stable colony formation. Because *Killer* is a novel apoptosis-inducing gene that appears to be regulated by p53, we further explore in this Example its regulation in p53-dependent G1-arrest versus apoptosis. Additionally, we examined the mechanism of regulation of
35 *Killer* by p53.

Materials and Methods

Cell Lines and Culture Conditions. The normal human lung fibroblast cell line WI38, the human ovarian

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cancer cell line SKOV3, the human breast cancer cell line SKBr3, and the human colon cancer cell line SW480 were obtained from the American Type Culture Collection (Rockville, MD). The human glioblastoma cell line GM
5 (Mercer et al., Proc. Natl. Acad. Sci. USA 87: 6166-6170, 1990) was obtained from Ed Mercer (Thomas Jefferson University).

Adenovirus Infection. The wild-type p53-expressing replication deficient adenovirus Ad-p53
10 and the LacZ-expressing adenovirus Ad-LacZ (El Deiry et al., 1993, *supra*) were obtained from Bert Vogelstein (Johns Hopkins University). Titering and infection with adenoviruses was carried out at an MOI of 50 as previously described (El Deiry et al., 1993, *supra*;
15 Blagoskonny & El Deiry, 1996, *supra*).

RNA extraction and Northern Blot. RNA extraction and Northern blot were as described in Example 1. The full-length (1.34 kb) human Killer cDNA probe was described in Example 1. The full-length human
20 p21WAF1/CIP1, MDM2 and p53 cDNA's are described elsewhere (El Deiry et al., 1993, *supra*). A human bax probe (Oltvai et al., Cell 74: 609-616, 1993; Miyashita et al., Oncogene 9: 1799-1805, 1994; Miyashita et al., Cell 80: 293-299, 1995) was provided by John Reed (Burnham
25 Institute).

Apoptosis assays. GM cells were infected by Ad-LacZ or Ad-p53. After 30 hrs, cells were stained with DAPI as previously described (Blagoskonny & El Deiry, 1996, *supra*).

Determination of messenger RNA stability.
30 SW480 human colon adenocarcinoma cells were infected by either Ad-LacZ or Ad-p53 for one hour using an MOI of 50 as previously described (Blagosklonny & El Deiry, 1996, *supra*; Meng et al, Clin. Cancer Res. 4: 251-259, 1998).
35 After an additional incubation at 37°C for 14 hrs., Actinomycin D was added to the cultures at a final concentration of 30 ng/ml. Total RNA was harvested at

- 40 -

different time points after Actinomycin D addition as indicated in Fig. 4, and Northern blotting was performed as described above.

5 Results

Killer expression is not increased in p53-dependent growth arrest. One of the major questions in elucidating p53 function has been to understand what determines whether cells undergo cell cycle arrest or
10 apoptosis following p53 activation. We have found a perfect correlation between induction of *Killer* expression and exposure of wild-type p53-expressing human cancer cells to apoptosis-inducing cytotoxic agents (Example 1). Following exposure to lethal doses of
15 agents such as adriamycin, etoposide, or ionizing radiation, only cells which contain wild-type p53 induced *Killer* expression. Cells with mutant or degraded p53 failed to upregulate *Killer* expression in response to genotoxic drugs. In order to investigate whether the
20 p53-dependent upregulation of *Killer* is specific to cells undergoing apoptosis, we used two well established systems where p53 induction leads only to cell cycle arrest.

The human glioblastoma cell line GM contains an
25 endogenous mutant p53 and an exogenous dexamethasone-inducible wild-type p53. Upon addition of dexamethasone to the media, it has been shown that these cells induce expression of wild-type p53 which in turn transcriptionally activates expression of p21, and the
30 cells then undergo cell cycle arrest in G1-phase and not apoptosis. Fig. 5A shows that upon addition of dexamethasone, the expected induction in p21 expression is observed, but there is no accompanying upregulation of *Killer* expression. These results suggest that cells
35 undergoing p53-dependent cell cycle arrest induce p21 as expected but do not induce expression of *Killer*, a putative mediator of p53-dependent apoptosis.

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In order to validate these observations in a different system, we chose to examine the expression levels of *p21* and *Killer* in WI38 normal human lung fibroblast cells either with no treatment or following exposure to ionizing radiation. It has been shown previously that the wild-type p53 containing WI38 lung fibroblasts respond to g- irradiation by undergoing a prolonged cell cycle arrest associated with *p21* upregulation, and not apoptosis. Following irradiation, *p21* expression was induced as expected but *Killer* expression was not increased (Fig. 5B). In fact at 10 Gy, *Killer* mRNA expression appeared to be inhibited. These results are consistent with the hypothesis that the p53-dependent induction of *Killer* is "apoptosis-specific" and not a general response in cells undergoing only p53-dependent cell cycle arrest.

Increased expression of *Killer* in cells undergoing p53-dependent apoptosis. To further investigate the hypothesis that p53-dependent upregulation of *Killer* expression may be a determinant of apoptosis induction, we infected GM cells with Ad-p53. GM cells were found to undergo apoptosis following Ad-p53 as compared to Ad-LacZ infection or dexamethasone treatment (Fig. 5A). Unlike the dexamethasone-treated GM cells which do not undergo p53-dependent apoptosis, the Ad-p53 infected cells upregulated expression of the *Killer* gene. We suspect that the higher levels of p53 achieved following Ad-p53 infection probably contribute to the apoptosis phenotype in GM cells. These results further correlate the p53-dependent induction of *Killer* with apoptosis.

We further investigated the kinetics of *Killer* upregulated expression following Ad-p53 as compared to Ad-LacZ infection of the SKOV3 human ovarian carcinoma cell line (Fig. 6B). Expression of *Killer* was detectably increased as early as 6 hours following Ad-p53 infection of the SKOV3 cells. This pattern of expression would be

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expected for genes involved in p53 mediated apoptosis which occurs over 1-3 days following Ad-p53 infection (Fig. 6A and data not shown).

Transcriptional and post-transcriptional control of TRAIL death receptor expression in p53-dependent apoptosis. We further explored the mechanism by which Killer is induced following p53 overexpression in cancer cells undergoing apoptosis. We infected SKOV3 cells (Fig. 7A) or GM cells (Fig. 7B) using either Ad-LacZ or Ad-p53 for one hour followed by a 20 hr incubation in the presence of the transcription inhibitor Actinomycin D. Expression of the p53 targets *p21*, *MDM2* and *Killer* was greatly increased in Ad-p53 as compared with Ad-LacZ infected SKOV3 cells in the absence of Actinomycin D (Fig. 7A, compare lanes 3 to lanes 1). In the presence of Actinomycin D, the p53-dependent upregulation of *p21*, *MDM2*, and *Killer* was abrogated (Fig. 7A, compare lanes 4 to lanes 2). These results suggest that *Killer*, *p21* and *MDM2* appear to be regulated by p53 at the level of transcription. p53 mRNA levels were also not decreased by Actinomycin D in Ad-p53 infected cells (Fig 7A).

We investigated the effect of Actinomycin D on the expression of *Killer* following infection of GM cells by Ad-p53 or Ad-LacZ (Fig. 3B). The p53- dependent upregulation of *Killer* expression (Fig. 7B lane 3 vs. 1, upper panel) was blocked by Actinomycin D in GM cells (lane 4 vs. 3).

The present invention is not limited to the embodiments specifically described above, but is capable of variation and modification without departure from the scope of the appended claims.

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania

(ii) TITLE OF THE INVENTION: NUCLEIC ACID ENCODING A NOVEL
10 CHEMOTHERAPY-INDUCED PROTEIN, AND METHODS OF USE

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: Dann, Dorfman, Herrell & Skillman, P.C.
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(C) CITY: Philadelphia
(D) STATE: Pennsylvania
(E) COUNTRY: USA
20 (F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
25 (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Not yet assigned
(B) FILING DATE: 10-JUL-1998
30 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/052,305
(B) FILING DATE: 11-JUL-1997
35 (A) APPLICATION NUMBER: US 60/054,710
(B) FILING DATE: 04-AUG-1997

(A) APPLICATION NUMBER: US 60/060,473
40 (B) FILING DATE: 30-SEP-1997

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45 (C) REFERENCE/DOCKET NUMBER: PENN J1638PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 215-563-4100
(B) TELEFAX: 215-563-4044
50 (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

55 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1139 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
60 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
65 (vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

- 44 -

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	CTAGCTCCCC	AGCAGAGAGC	GGCCCCACAA	CAAAAGAGGT	CCAGCCCCCTC	AGAGGGATTG	240
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 411 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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- 45 -

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(2) INFORMATION FOR SEQ ID NO:3:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 468 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: internal
- 35 (vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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- 46 -

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          290          295          300
10 Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met Glu Ser Gln Glu Pro
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Ala Asp Leu Thr Gly Val Thr Val Gln Ser Pro Gly Glu Ala Gln Cys
          325          330          335
Leu Leu Gly Pro Ala Glu Ala Glu Gly Ser Gln Arg Arg Arg Leu Leu
15          340          345          350
Val Pro Ala Asn Gly Ala Asp Pro Thr Glu Thr Leu Met Leu Phe Phe
          355          360          365
Asp Lys Phe Ala Asn Ile Val Pro Phe Asp Ser Trp Asp Gln Leu Met
370          375          380
20 Arg Gln Leu Asp Leu Thr Lys Asn Glu Ile Asp Val Val Arg Ala Gly
385          390          395          400
Thr Ala Gly Pro Gly Asp Ala Leu Tyr Ala Met Leu Met Lys Trp Val
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25          420          425          430
Glu Arg Met Glu Glu Arg His Ala Lys Glu Lys Ile Gln Asp Leu Leu
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(2) INFORMATION FOR SEQ ID NO:4:

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35  (i) SEQUENCE CHARACTERISTICS:
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      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
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      (ii) MOLECULE TYPE: peptide
      (iii) HYPOTHETICAL: NO
      (iv) ANTISENSE: NO
      (v) FRAGMENT TYPE: internal
45  (vi) ORIGINAL SOURCE: Homo sapiens

```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

50  Arg Trp Lys Glu Phe Val Arg Thr Leu Gly Leu Arg Glu Ala Glu Ile
     1      5      10      15
Glu Ala Val Glu Val Glu Ile Gly Arg Phe Arg Asp Gln Tyr Glu
     20      25      30
Met Leu Lys Arg Trp Arg Gln Gln Gln Pro Ala Gly Leu Gly Ala Val
     35      40      45
55  Tyr Ala Ala Leu Glu Arg Met Gly Leu Asp Gly Cys Val Glu Asp Leu
     50      55      60
Arg
65

```

60 (2) INFORMATION FOR SEQ ID NO:5:

```

65  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 68 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

```


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(ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: internal
 5 (vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10 Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile
 1 5 10 15
 Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys Val
 20 25 30
 Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala Tyr
 35 40 45
 15 Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala
 50 55 60
 Glu Lys Ile Gln
 65

20 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 69 amino acids
 (B) TYPE: amino acid
 25 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 30 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: internal
 (vi) ORIGINAL SOURCE: homo sapiens

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile
 1 5 10 15
 Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr
 20 25 30
 40 Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr
 35 40 45
 Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys
 50 55 60
 45 Leu Glu Asp Ile Glu
 65

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 67 amino acids
 (B) TYPE: amino acid
 50 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
 (iii) HYPOTHETICAL: NO
 55 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE: internal
 (vi) ORIGINAL SOURCE: Homo sapiens

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Trp Lys Arg Phe Gly Arg Ala Leu Asp Leu Gln Glu Asn Asp Leu
 1 5 10 15
 65 Tyr Leu Ala Glu Gln His Glu Arg Val Ser Cys Glu Pro Arg Tyr Gln
 20 25 30
 Met Leu Asn Thr Trp Leu Asn Gln Gln Gly Ser Lys Ala Ser Val Asn

- 48 -

35 40 45
 Thr Leu Leu Glu Thr Leu Pro Arg Ile Gly Leu Ser Gly Val Ala Asp
 50 55 60
 Ile Ile Ala
 5 65

(2) INFORMATION FOR SEQ ID NO:8:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE: synthetic

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
 CCCAAGCTTC CGCCATGGAA CAACGGGGAC 30

(2) INFORMATION FOR SEQ ID NO:9:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 30 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE: synthetic

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
 CGCGGATCCT TAGGACATGG CAGAGTCTGC 30

(2) INFORMATION FOR SEQ ID NO:10:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 50 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
 CGCGGATCCG TCCTCAATCT TCTGCTTGG 29

(2) INFORMATION FOR SEQ ID NO:11:

65 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
5 (vi) ORIGINAL SOURCE: synthetic
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
10 CCCAAGCTTC CACCATGGTT GCAGCCGTAG TCTTG 35
(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
15 (A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
20 (iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic
25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
CCCAAGCTTC CACCATGGAC TCCTGGGAGC CGC 33
(2) INFORMATION FOR SEQ ID NO:13:
30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
35 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
40 (v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
45 CGCGGATCCG TCCTCAATCT TCTGCTTG 29

- 50 -

What is claimed is:

1. An isolated nucleic acid molecule comprising an open reading frame of part or all of a gene located on human chromosome 8p21, which encodes part or
5 all of a mammalian p53-inducible, apoptosis-mediating protein, which comprises an amino-terminal extracellular receptor domain, a transmembrane domain, and a death domain for protein-protein interactions.
- 10 2. The nucleic acid molecule of claim 1, which encodes a human protein.
3. The nucleic acid molecule of claim 2, which
15 is DNA.
4. The DNA molecule of claim 3, which is a cDNA comprising a sequence approximately 1.2 kilobase pairs in length that encodes said p53-inducible, apoptosis-mediating protein.
- 20 5. The DNA molecule of claim 3, which is a gene, the exons of which comprise said open reading frame.
- 25 6. The nucleic acid molecule of claim 2, which encodes a polypeptide having a sequence substantially the same as SEQ ID NO:2.
- 30 7. The nucleic acid molecule of claim 6, which encodes SEQ ID NO:2.
8. The nucleic acid molecule of claim 8, which comprises SEQ ID NO:1.
- 35 9. A vector for transformation of a mammalian cell, which comprises the nucleic acid molecule of claim 1.

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10. A mammalian cell transformed with the vector of claim 9.

11. An oligonucleotide between 10 and 100
5 nucleotides in length, that specifically hybridizes with a pre-determined portion of the nucleic acid molecule of claim 1.

12. An isolated nucleic acid molecule having a
10 sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural
mutant of SEQ ID NO:1;

c) a sequence hybridizing with part
15 or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1; and

d) a sequence encoding part or all
of a polypeptide having amino acid SEQ ID NO:2.

20

13. A protein produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on human chromosome 8p21, said protein being a p53-induced
25 apoptosis-mediating protein having an amino-terminal extracellular receptor domain, a transmembrane domain, and a death domain for protein-protein interactions.

14. The protein of claim 13, comprising an
30 amino acid sequence substantially the same as SEQ ID NO:2.

15. The protein of claim 13, produced by expression of an open reading frame having a sequence
35 substantially the same as SEQ ID NO:1.

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16. Antibodies immunologically specific for the protein of claim 13.

17. A protein produced by expression of a
5 sequence selected from the group consisting of:
a) SEQ ID NO:1;
b) an allelic variant or natural
mutant of SEQ ID NO:1;
c) a sequence hybridizing with part
10 or all of SEQ ID NO:1 or its complement and encoding a
polypeptide substantially the same as part or all of a
polypeptide encoded by SEQ ID NO:1; and
d) a sequence encoding part or all
of a polypeptide having amino acid SEQ ID NO:2.

15

18. Antibodies immunologically specific for the protein of claim 17.

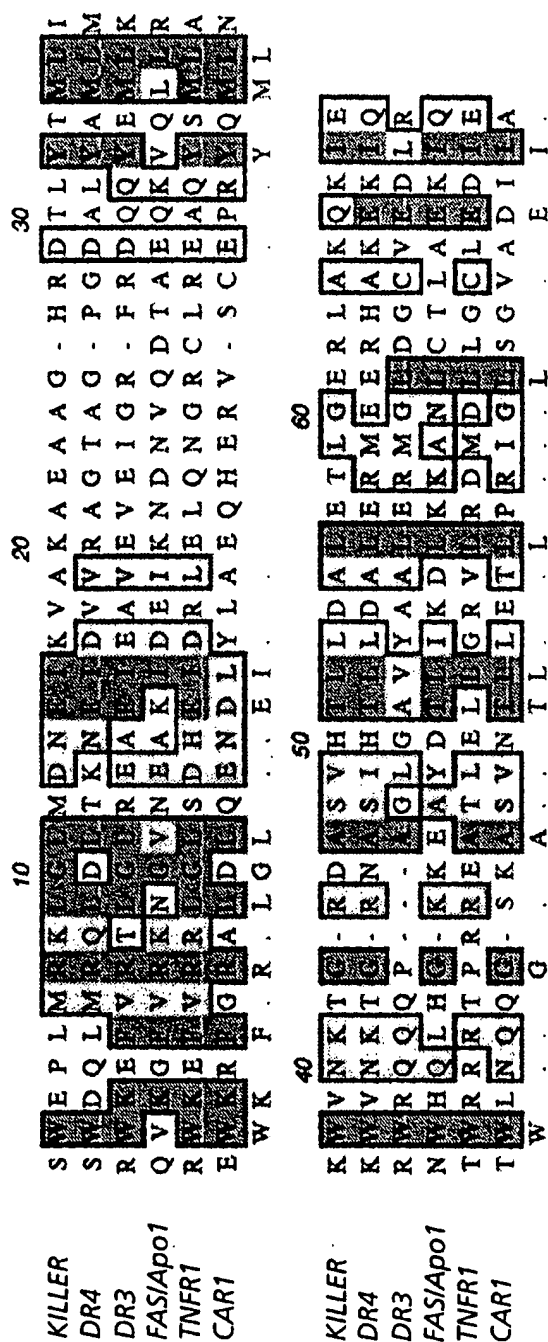
19. A non-human genetically engineered mammal,
20 which is unable to produce a functional protein normally
produced by expression of an isolated nucleic acid
molecule comprising part or all of an open reading frame
of a gene located on human chromosome 8p21, said protein
being a p53-induced apoptosis-mediating protein having an
25 amino-terminal extracellular receptor domain, a
transmembrane domain, and a death domain for protein-
protein interactions.

20. The genetically engineered mammal of claim
30 19, which is a mouse.

1/1
 ATG GAA CAA CCG GGA CAG AAC GGC GGC GCT TCG GGG GGC AAA AGG CAC GGC CCA CCA CCG GGA GGC AGG CCT
 M E Q R G Q N A P A A S G A R K R H G P G P R E A R G A R P
 91/31
 GCG CCC CCG GTC CCC AAG ACC CTT GTG CTC GTT GTG GGC GGC GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT CTG ATC ACC CAA CAA GAC
 G P R V P K T L V L V V A A V L L L V S A E S A L I T Q Q D
 181/61
 CTA GCT CCC CAG CAG AGA CCG GGC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG TTG TCA CCA CCA CCG GGA GGC AGG CCT
 L A P Q Q R A A P Q Q K R S S P S E G L C P P G H H I S E D
 271/91
 GGT AGA GAT TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC TGG AAT GAC CTC CTT TTC TGC TTG CCG CCG ACC AGG TGT GAT
 G R D C I S C K Y G Q D Y S T H W N D L L F C L R R T R C D
 361/121
 TCA GGT GAA GTG GAG CTA AGT CCC TCC ACC ACC AGA AAC ACA GTG TGT CAG TCC GAA GAA GGC ACC TTC CCG GAA GAA GAT TCT CCT
 S G E V E L S P C T T T R N T V C Q C E E G T F R E E D S P
 451/151
 GAG ATG TGC CCG AAG TGC CCG ACA GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA CCC TGG AGT GAC ATC GAA TGT GTC CAC
 E M C R K C R T G C P R G M V K V G D C T P W S D I E C V H
 541/181
 AAA GAA TCA GGC ATC ATC GGA GTC ACA GTT GCA GGC GTA GTC TTG ATT GTG GCT GTG TTT TGC AAG TCC TTA CTG TGG AAG AAA
 K E S G I I I G V T V A A V V L I V A V F V C K S L L W K K
 631/211
 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT GGC GAC CCT GAG CCG GTG GAC AGA AGA TCA CAA CCA CCT GGG GCT GAG GAC
 V L P Y L K G I C S G G G G D P E R V D R S S Q R P G A E D
 721/241
 AAT GTC CTC AAT GAG ATC GTG AGT ATC TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC CAG GAG CCA CCA GAG CCA ACA GGT
 N V L N E I V S I L Q P T Q V P E Q E M E V Q E P A E P T G
 811/271
 GTC AAC ATG TTG TCC CCC GGG GAG TCA GAG CAT CTG GAA CCG GCA GAA GCT GAA AGG TCT CAG AGG AGG CTG CTG GTT CCA GCA
 V N M L S P G E S E H L L E P A E A E R S Q R R R L L V P A
 901/301
 AAT GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT GAC TTT CCA GAC TTG GTG CCG TTT GAC TCC TGG GAG CCG CTC ATG AGG
 N E G D P T E T L R Q C F D D F A D L V P F D S W E P L M R
 991/331
 AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG TAC ACG ATG CTG ATA AAG TGG
 K L G L M D N E I K V A K A E A A G H R D T L Y T M L I K W
 1081/361
 GTC AAC AAA ACC GGG CGA GAT GAT GGC TCT GTC CAC ACC CTG CTG GAT GCT TTG GAG ACG CTG GGA GAG AGA CTT GCG AAG CAG AAG ATT GAG
 V N K T G R D A S V H T L L D A L E T L G E R L A K Q K I E
 1171/391
 GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TCA GAA GGT AAT GCA GAC TCT GCG ATG TCC TAA
 D H L L S S G K F M Y L E G N A D S A M S

FIG. 1

FIG. 2A



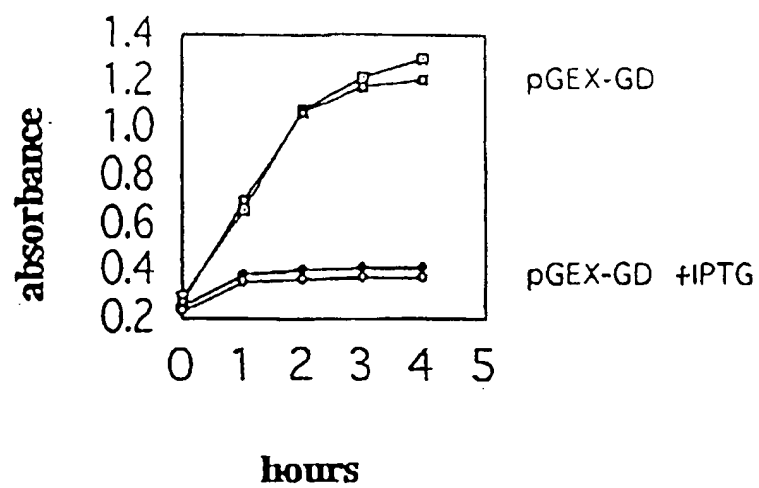
**FIG. 4**

FIG. 5A

GM Cells

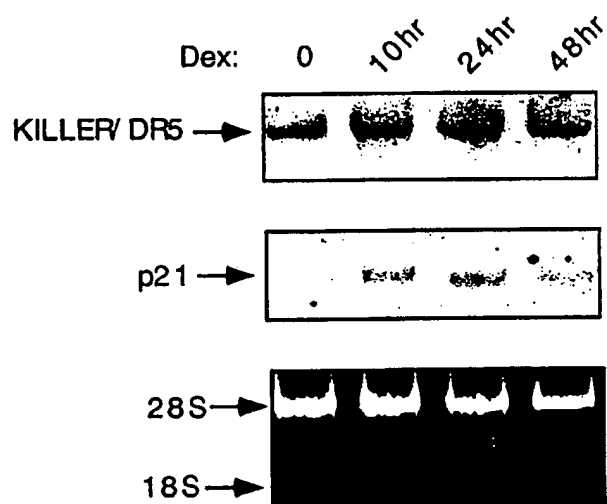


FIG. 5B

WI38 Cells

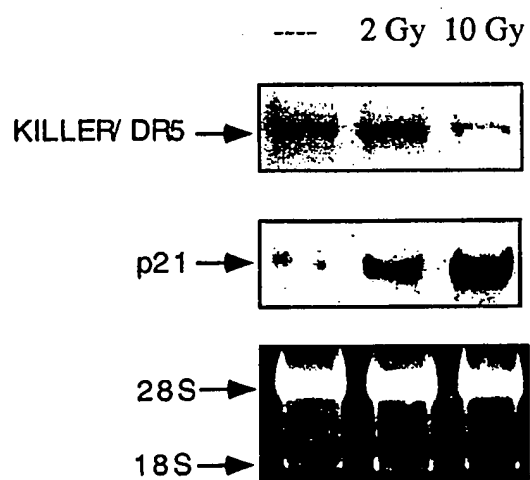


FIG. 6A

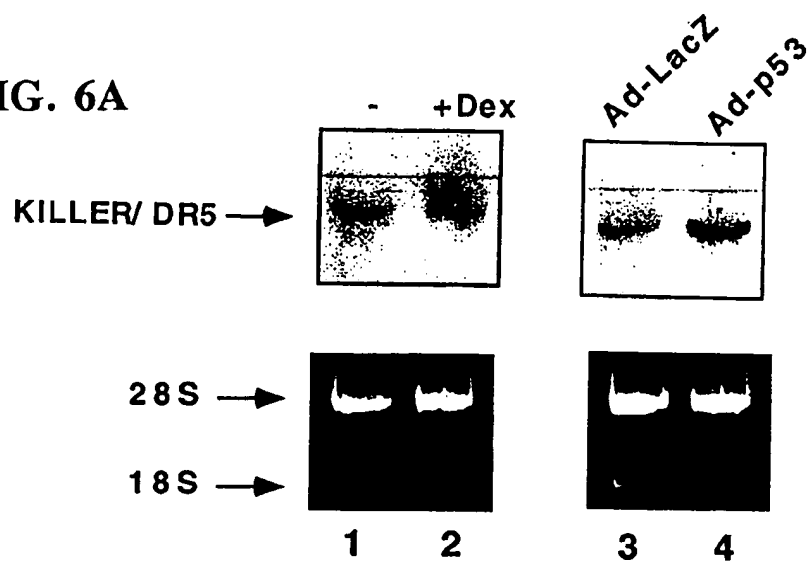


FIG. 6B

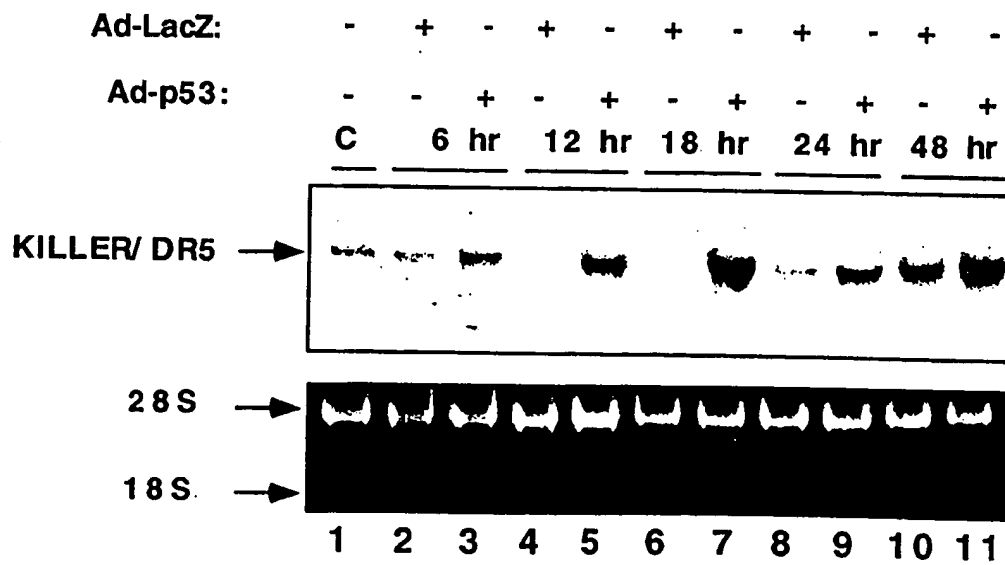


FIG. 7A

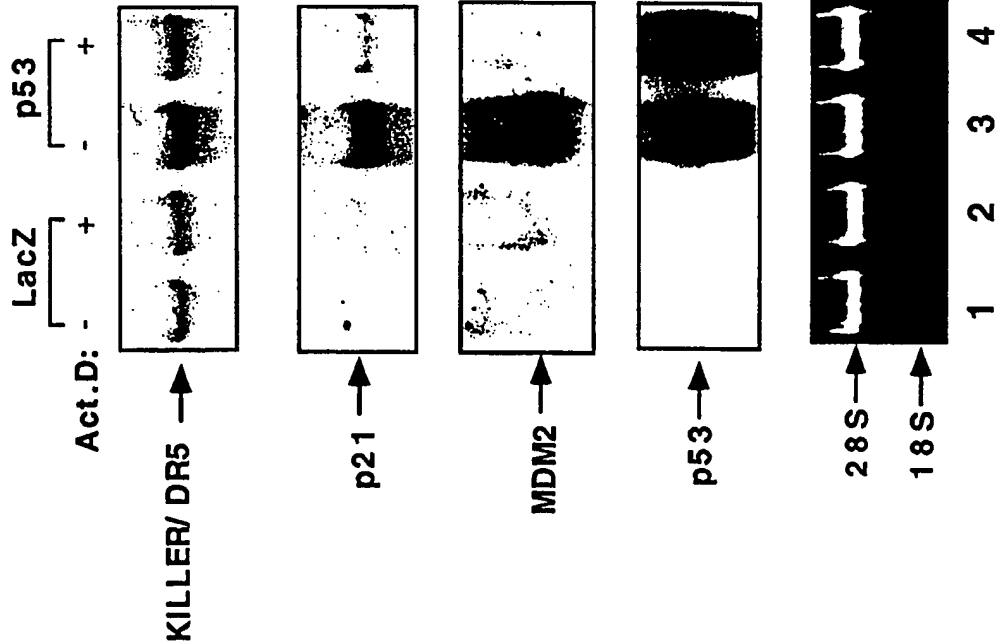
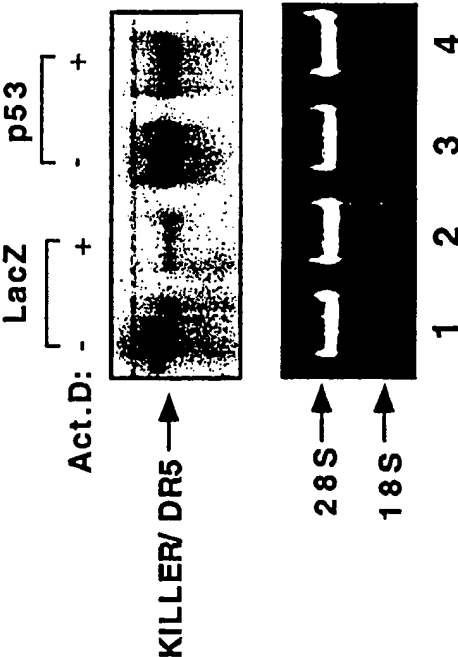


FIG. 7B



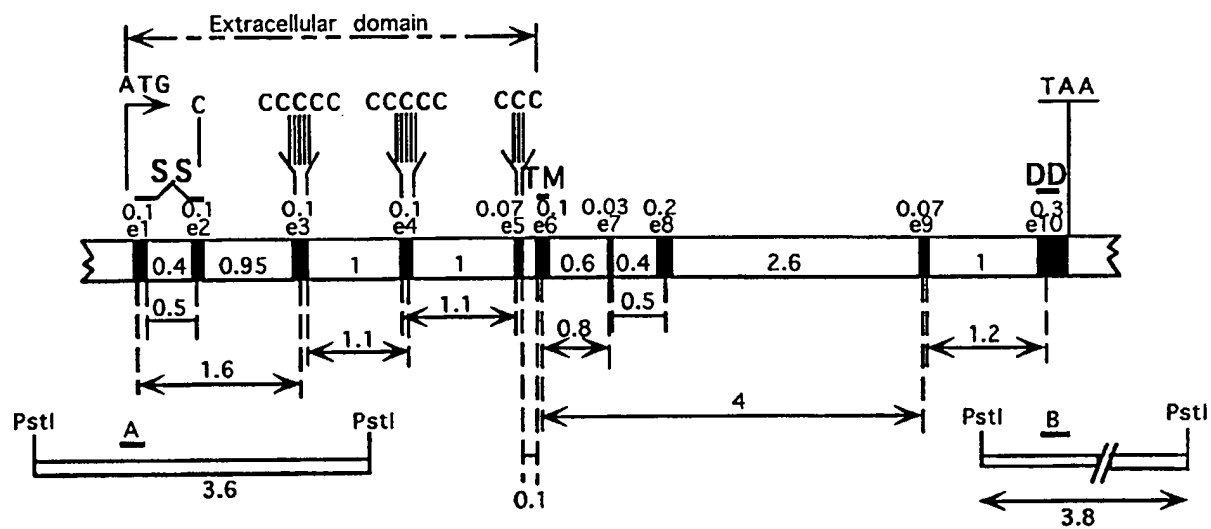


FIG. 8

FIG. 9

10 20 30 40 50 60
TGGCTCGTCTGTTCCCTCTACGGCCCCCTCCGCATGGCGCTCCACCCCAAGTCTTCAGAAATT
ACCGAGCAGACAAGGAGATGCCGGGAGGCGTACCGCGAGGTGGGGTCAGAAGTCTTTAA

70 80 90 100 110 120
TCTGCCTCAAGCTCAGGGAGCTGGTACTGAGGTACAGGTAGGAGAAGGTGCAGCCTTCG
AGACGGAGTTCGAGTCCCTCGACCATGACTCCATGTCCATCCTCTTCCACGTCCGAGACG

130 140 150 160 170 180
TGAGTGGAGGACATGTGCCCTGGGGATCCAGAGTCTANAGTGAAGTACACAGCCCTGCA
ACTCACCTCCTGTACACGGGACCCCTAGGTCTCAGATNTCACTGACTGTGGTCCGGACGT

190 200 210 220 230 240
ATTTGTCTCCTAGACCTGTTAACAGAGCCTGCAGAAATTACACAGGGCTGCTGAAGAT
TAAACAGGAGGATCTGGACAATTGTCTCGGACGTCTTCAAATGTGTCCCGACGACTTCTA

250 260 270 280 290 300
TTTTATCCCCCATTTTACAGGAAGGAGCTGGGGCCATACCANANTCAGAGCTGGACCGG
AAAATAGGGGAGTAAAATGTCCTTCCTCGACCCCGGTATGGTNTNAGTCTCGACCTGGCC

310 320 330 340 350 360
GANCACGGCAGGGCCCANCCATCCACAGCCTCCAATTTCACTACTCTCTAACAACCTGAN
CTNGTGCCGTCCCGGGTNGGTAGGTGTGGGAGGTAAAGTCATGAGAGATTGTTGGACTN

370 380 390 400 410 420
ANCCTGANCCCTTCGGATCTTCTTGATTCCCTACTTTGATAAANAAAACGATGGGGTTGGAA
TNGGACTNNGAAGCCTAGAAGAACTAAGGATGAAACTATTTNTTTTCTACCCCAACCTT

430 440 450 460 470 480
AGACTGGCATANAAATTGACCANTCCGGCATTGACANAACCANGAACTGGGGTCCGGAA
TCTGACCGTATNTTTAACCTGGTNAGGCCGTAAGTGTNTTGGTNCCTTGACCCCAAGCCCTT

490 500 510 520 530 540
CTGAACCTCACTCCANGCTCATCTGAATTCACCTGACANCCTGAAGATGGAAAACCTATTT
GACTTGAGTGAAGTNCAGTAGACTTAAGTGGACTGTNGGACTTCTACCTTTGAATAAA

550 560 570 580 590 600
CCATTAAACANGCATNGCGGTGGCANGGACTGGCCCCANCTGAACACGGTTTTCGAATTA
GGTAATGTNCGTANCGCCAACCGTNCCTGACCGGGTNGACTTGTGCCAAAAGGTTAAT

610 620 630 640 650 660
CACANCTGGGAATTAANTCTTCTCTGCCGCCCTCCCTGTTATTCTTGCCCTGGAATCCCC
GTGTNGACCCCTTAATTNAGAAGAGACGCCGGGAGGGGACAATAAGAACGGACCTTAGGGG

670 680 690 700 710 720
CCAGTCATCTACTCCAGTACCAAAAATCCTGTATTCTTGAAAATCCGTAGTACTGAGAG
GGTCAGTAGATGAGGGTCATGGTTTTTAGGACATAAGAACTTTTAGGCATCATGACTTC
_10_____20_8 TO 1109 OF P1-KILLER-1_50_____60_>

730 740 750 760 770 780
GTGACAGCTTGCTGGCAGTCTCAACAGCCCTCGCTCGCTCTCGGCGCCTCCTCTGCCTG
CACTGTGGAACGACCGTCAGGAGTTGTCCGGAGCGAGCGAGAGCCGCGGAGGAGACGGAC
_____70_____8_8 TO 1109 OF P1-KILLER-1_110_____120_>

790 800 810 820 830 840
GGCTCCCACTTTGGCGGCACCTTGANGAGCCCTTCAGCCCAACCGCTGCACTGTGGGAGCCC
CCGAGGGTGAAACCGCCGTGAACNTCTCGGAAGTGGGTGGCGACGTGACACCCTCGGG

FIG. 9 - cont'd

_____130_____14_8 TO 1109 OF P1-KILLER-1_____170_____180____>
 850 860 870 880 890 900
 TTTTCTGGGCTGGCCAAGGCTGGAGCCCGCTCCCTCAGCTTGACGGAGCTGTGGANGGA
 AAAAGACCCGACCGGTTCCGACCTCGGGCGAGGGAGTCGAACGTGCCTCCACACCTNCCT
 _____190_____20_8 TO 1109 OF P1-KILLER-1_____230_____240____>
 910 920 930 940 950 960
 GAGGCGCGAGCGGGAACCGGGGCTGCGCGCGCGCACTTGCGGGCCAGCTGGAGTTCCGG
 CTCCGCGCTCGCCCTTGCCCCGACGCGCGCGCGTGAACGCCCCGGTCGACCTCAAGGCC
 _____250_____26_8 TO 1109 OF P1-KILLER-1_____290_____300____>
 970 980 990 1000 1010 1020
 GTGGGCGTGGGCTCGGCGGGCCCTGCACTCGGAGCAGCCGGCCGGCCCTGCCGGCCCCGG
 CACCCGACCCGAGCCGCCCGGACGTGAGCCTCGTCGGCCGGCCGGGACGGCCGGGGCC
 _____310_____32_8 TO 1109 OF P1-KILLER-1_____350_____360____>
 1030 1040 1050 1060 1070 1080
 GCAATGANGGGTTTAGCACCCGGGCCANCGGCTGCGGANGGTGTACTGGGTCCCCCANCA
 CGTTACTNCCCAAATCGTGGGCCCCGTNGCCGACGCTNCCACATGACCCAGGGGGTNGT
 _____370_____38_8 TO 1109 OF P1-KILLER-1_____410_____420____>
 1090 1100 1110 1120 1130 1140
 ATGCCCGCCCACTGGCGCTGCGCTTGATTTCTCGCCGGGCTTAGCTGCCTTCCCCGACGG
 TACGGCCGGGTGACCGCGACGCGAACTAAAGAGCGGCCCCGGAATCGACGGAAGGGCTGCC
 _____8 TO 1109 OF P1-KILLER-1_____>
 1150 1160 1170 1180 1190 1200
 GCAGGCTCGGGACCGCCATGCTGAGCCTCTCACCCCTCCGTGGGCTCCTGTGCAGCC
 CGTCCCGAGCCCTGGCGGTACGGACTCGGAGAGTGGGGGAGGCACCCGAGGACACGTCCG
 1210 1220 1230 1240 1250 1260
 GGAGCCTCCCCGACGAGCACCTCCCCCTGCTCCAGGGCGCCAGTCCCATCGACCACCCA
 CCTCGGAGGGGCTGCTCGTGGAGGGGACGAGCTCCCGCGGGTCAGGGTAGCTGGTGGGT
 1270 1280 1290 1300 1310 1320
 AGGGCTGAGGAGTGGCGGCGCACGGCGCGGGAGCACGGCGCGGGACTGGCAGGCAGCTCC
 TCCCGACTCCTACGCCCCGCGTCCCGCGCCCTCGTGCCGCGCCCTGACCGTCCGTCGAGG
 1330 1340 1350 1360 1370 1380
 ACCTGCAACCCCGGTGCGGGATCCACTGGGTGACGACACCTGGGCTCCTGAGTCTGGTGG
 TGGACGTTGGGGCCACGCCCTAGGTGACCCACTGCTGTGGACCCGAGGACTCAGACCACC
 1390 1400 1410 1420 1430 1440
 GGACGTGGAGAACCTTTATGTTTAGCTAAGGGATTGTAAATACACCAATTGGCTACTGTGT
 CCTGCACCTCTTGGAATACAAATCGATTCCCTAACATTTATGTGGTTAACCGTGACACA
 1450 1460 1470 1480 1490 1500
 ATCTAGCTCAAGTTTGTAAACACACCAATCAGCACCGTGTGTCTAGCTCAGGGTTTGTG
 TAGATCGAGTTCAAAACATTTGTGTGGTTAGTCGTGGCACACAGATCGAGTCCCAAACAC
 1510 1520 1530 1540 1550 1560
 AATGCACCAATTGACACTCTGTATCTAGCTAGTCTGGTGGGGCTTGGAGAACCTTTGTG
 TTACGTGGTTAACTGTGAGACATAGATCGATCAGACCACCCCGGAACCTCTTGGAAACAC
 1570 1580 1590 1600 1610 1620
 TCCCACTCTGTATCTAGCTAATCTGGTGGGGAAGTGGAGAACATTTGTGTCTAGCTCAG

FIG. 9 - cont'd

AGGTGTGAGACATAGATCGATTAGACCACCCCTTCACCTCTTGTAACACAGATCGAGTC

1630 1640 1650 1660 1670 1680
GGATTGTAAACCACCAATCAGCGCCCTGTCAAAACAGACCACTCCGCTCTACCAATCAGC
CCTAACATTTGGTGGTTAGTCGCGGGACAGTTTGTCTGGTGAGGCGAGATGGTTAGTCG

1690 1700 1710 1720 1730 1740
AGGGTGTGGGTGGGGCCANGATAAGAGAATAAAAGCAGGCTGCCAGAGCCAGCAGTGGTA
TCCCACACCCACCCCGGTNCTATTCTCTTATTTTCGTCCGACGGTCTCGGTCTCACCAT

1750 1760 1770 1780 1790 1800
ACACTGTGGAAGTTTCTTCTTTCCCTCTGCAATAAATCTTGCTACTTGCTCACTCTTTG
TGTGACACCTTCAAAGGAAGAAAGGGAGACGTTATTTAGAACGATGAACGAGTGAGAAAC
____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_0____>
____10____1 TO 815 OF WU/9/12/CHOP_50____>

1810 1820 1830 1840 1850 1860
GGTCCACANTGCTTTATGAGCTTGAACACTCACCTTGAAGGTCTGCAGCTTCACTCTT
CCAGGTGTNACGGAAATACTCGAACATTGTGAGTGGAACTTCCAGACGTCGAAGTGAGAA
____60____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_110____>
____60____70____1 TO 815 OF WU/9/12/CHOP_00____110____>

1870 1880 1890 1900 1910 1920
GAAGCCAGCGAGACCCGAGCCACCGGGAGGAAAGAACAACCTCCAGACCCACTGCCTTA
CTTCGGTCTGCTCTGCTCGGGTGGCCCTCCTTTCTTGTGAGGTCTGGGTGACGGAAT
____120____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_170____>
____120____130____1 TO 815 OF WU/9/12/CHOP_60____170____>

1930 1940 1950 1960 1970 1980
AGAGCTGTAACACTCACTCGGAAGGTCTGCAGCTTCACTCCTGAGCCAGTGAGACCCACGA
TCTCGACATTGTGAGTGACCCCTTCCAGACGTCGAAGTGAGGACTCGGTCACTCTGGTGCT
____180____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_230____>
____180____190____1 TO 815 OF WU/9/12/CHOP_20____230____>

1990 2000 2010 2020 2030 2040
ACCCACCAGAAGGAAGAACTCCGAACACATCCGAACATCAGAAGGAACAAACTCCAGAC
TGGGTGGTCTTCTTCTTTGAGGCTTGTGTAGGCTTGTAGTCTTCTTGTGAGGTCTG
____240____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_290____>
____240____250____1 TO 815 OF WU/9/12/CHOP_80____290____>

2050 2060 2070 2080 2090 2100
ACGCCGCCTTTAAGAACTGTAAACTCACCGCGAGGGTCCGAGGCTTCATTCTTGAAGGC
TGCGGCGGAAATCTTGACATTGTGAGTGGCGCTCCAGGCTCCGAAGTAAGAAGTTCG
____300____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_350____>
____300____310____1 TO 815 OF WU/9/12/CHOP_40____350____>

2110 2120 2130 2140 2150 2160
AGTGAGACCAAGAACCACCAATTCGGGACACAGTACCATGAAGGAATGAAATACATTA
TCACTCTGGTTCTTGGGTGGTTAAGGCCTGTGTATGGTACTTCTTACTTTTATGTATT
____360____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_410____>
____360____370____1 TO 815 OF WU/9/12/CHOP_00____410____>

2170 2180 2190 2200 2210 2220
CAATGTGATGTATCATGTTTATTTCTAGACTAGTGACAAATGAAAGCTAAGTGTAGCA
GTTACACTACATAGTACAAATATAAGGATCTGATCACTGTTTACTTTCGATTACATCGT
____420____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_470____>
____420____430____1 TO 815 OF WU/9/12/CHOP_60____470____>

[illegible]

FIG. 9 - cont'd

CAACTCATTTCCTCCCAAGTTTCGGTGCCTGTCTGGCGCGGACAGGACCCAGAAACAAAC
GTTGAGTAAAGGGGGTTCAAAGCCACGACAGGACCGCGCCTGTCTGGGTCTTTGTTTG
1020____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_070____>

2830 2840 2850 2860 2870 2880
CACAGCCCCGGGGCAGCGCCAGGGCGAAGGTTAGTTCCTTTCCCTTCCCTTCCCAACTT
GTGTCCGGCCCCCGTCGCGGTCCCGCTTCCAATCAAGGAAAGGAAGGGGAGGGGTTGAA
1080____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_130____>

2890 2900 2910 2920 2930 2940
GGAAGGGGGTTTGCCTGAGGATTGCGTTGACGAGACTCTTATTTATTGTCACCAACCTGTGG
CCTTCCCAACAGCTCTCTAACGCAACTGCTCTGAGAATAAATAACAGTGGTTGGACACC
1140____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_190____>

2950 2960 2970 2980 2990 3000
TGAATTTGCAGTTGCACATTGGATCTGATTCGCCCCGCCCCGAATGACGCTGCCCGGAG
ACCTTAAACGTCACGTGTAACTAGACTAAGCGGGGCGGGGCTTACTGCGACGGGCCTC
1200____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_250____>

3010 3020 3030 3040 3050 3060
GCAGTGAAAGTACAGCCGCCCCGCCCAAGTCAGCCTGGACAACATAAATCAGCACGCGG
CGTCACCTTCATGTGCGCGGGGCGGGGTTCAAGTCGGACCTGTTGTATTTAGTCGTGCGCC
1260____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_310____>

3070 3080 3090 3100 3110 3120
CCGGAGAACCCTCGCAATCTCTGCGCCCAAAAATAACCGACGATGCCCGATCTACTTTA
GGCCTCTTGGCGGCTTAGAGACGCGGGTCTTTTATGTGGCTGCTACGGGCTAGATGAAAT
1320____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_370____>

3130 3140 3150 3160 3170 3180
AGGGCTGAAACCCACGGGCGCTGAGAGACTATAAGCGTTCCCTACCGCCTGGAAACAC
TCCCGACTTTGGGTGCCCCGACTCTCTGATTTCTCGCAAGGGATGGCGGTACCTTGTG
1380____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_430____>

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3190 3200 3210 3220 3230 3240
GGGGACAGAACGCCCCGGCCGCTTCGGGGGCCCCGAAAAGGCACGCCCCAGGACCCAGGG
CCCCGTCTTGCGGGGCCGCGAAGCCCCGGGCGCTTTTCCGTGCCGGGTCTGGGTCCC
1440____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_490____>

3250 3260 3270 3280 3290 3300
AGGCGCGGGGAGCCAGGCCTGGGCCCCGGGTCCCAAGACCCCTTGTGCTCGTTGTGCGCCG
TCCGCGCCCCCTCGGTCCGACCCGGGGCCCCAGGGGTTCTGGGAACACGAGCAACAGCGCG
1500____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_550____>

3310 3320 3330 3340 3350 3360
CGGTCTGCTGTTGGTCTCAGCTGAGTCTGCTCTGATCACCCAACAAGACCTAGCTCCCC
GCCAGGACGACAACCAGAGTCGACTCAGACGAGACTAGTGGGTGTTCTTGGATCGAGGGG
1560____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_610____>

3370 3380 3390 3400 3410 3420
AGCAGAGAGCGGCCCCACAACAAAAGAGGTCCAGCCCCCTCAGAGGGATTGTGTCCACCTG
TCGTCTCTCGCCGGGGTGTGTTTCTCCAGGTCGGGGAGTCTCCCTAACACAGGTGGAC
1620____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_670____>

3430 3440 3450 3460 3470 3480

FIG. 9 - cont'd

GACACCATATCTCAGAAGACGGTAGAGATTGCATCTCCTGCAAATATGGACAGGACTATA
CTGTGGTATAGAGTCTTCTGCCATCTCTAACGTAGAGGACGTTTATACCTGTCCTGATAT
1680_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_730_____>

3490 3500 3510 3520 3530 3540
GCACTCACTGGAATGACCTCCTTTTCTGCTTGCGCCGACCCAGGTGTGATTCAAGTGAAG
CGTGACTGACCTTACTGGAGGAAAAGACGAACGCGCGTGGTCCACACTAAGTCCACTTC
1740_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_790_____>

3550 3560 3570 3580 3590 3600
TGGAGCTAAGTCCCTGCACCACGACCAGAAACACAGTGTGTCAGTGCAGAAGAAGGCACCT
ACCTCGATTACAGGACGTGGTGTCTGCTTTGTGTACACAGTCACGCTTCTTCCGTGGA
1800_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_850_____>

3610 3620 3630 3640 3650 3660
TCCGGGAAGAAGATTCTCCTGAGATGTGCCGGAAGTGCCGCACAGGGTGTCCAGAGGGA
AGGCCCTTCTTCTAAGAGGACTCTACACGGCCTTCACGGCGTGTCCACAGGGTCTCCCT
1860_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_910_____>

3670 3680 3690 3700 3710 3720
TGGTCAAGGTGGTGATTGTACACCCTGGAGTGACATCGAATGTGTCCACAAAGAATCAG
ACCAGTTCACGCCACTAACATGTGGGACCTCACTGTAGCTTACACAGGTGTTTCTTAGTC
1920_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_970_____>

3730 3740 3750 3760 3770 3780
GCATCATCATAGGAGTCACAGTTGCAGCCGTAGTCTTGATTGTGGCTGTGTTTGTGTTGCA
CGTAGTAGTATCCTCAGTGTCAACGTTCGGCATCAGAACTAACACCGACACAAACAAACGT
1980_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_030_____>

3790 3800 3810 3820 3830 3840
AGTCCCTTACTGTGGAAGAAAGTCCTTACCTGAAAGGCATCTGCTCAGGTGGTGGTG
TCAGGAATGACACCTTCTTTCAGGAAGGAATGGACTTCCGTAGACGAGTCCACCACCAC
2040_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_090_____>

3850 3860 3870 3880 3890 3900
GGGACCTGAGCGTGTGGACAGAAGCTCACAACGACCTGGGGCTGAGGACAATGTCCTCA
CCCTGGGACTCGCACACCTGTCTTCGAGTGTGCTGGACCCGACTCCTGTTACAGGAGT
2100_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_150_____>

3910 3920 3930 3940 3950 3960
ATGAGATCGTGAGTATCTTGCAGCCCACCCAGGTCCCTGAGCAGGAAATGGAAGTCCAGG
TACTCTAGCACTCATAGAACGTCCGGTGGGTCCAGGACTCGTCTTTACCTTCAGGTCC
2160_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_210_____>

3970 3980 3990 4000 4010 4020
AGCCAGCAGAGCCAACAGGTGTCAACATGTTGTCCCCGGGGAGTCAGAGCATCTGCTGG
TCGGTCTGTCGGTTGTCCACAGTTGTACAACAGGGGGCCCCCTCAGTCTCGTAGACGACC
2220_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_270_____>

4030 4040 4050 4060 4070 4080
AACCCGCAGAAGCTGAAAGGTCTCAGAGGAGGAGGCTGCTGGTTCCAGCAAATGAAGGTG
TTGGCCGTCTTCGACTTTCAGAGTCTCCTCCTCCGACGACCAAGGTCGTTTACTTCCAC
2280_____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_330_____>

4090 4100 4110 4120 4130 4140
ATCCCACTGAGACTCTGAGACAGTGCTTCGATGACTTTGCAGACTTGGTGCCCTTTGACT
TAGGGTGACTCTGAGACTCTGTACGAAGCTACTGAAACGTCTGAACCACGGGAAACTGA

FIG. 9 - cont'd

2340____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_390____>
4150 4160 4170 4180 4190 4200
CCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAG
GGACCCCTCGGCGAGTACTCCTTCAACCCGGAGTACCTGTTACTCTATTTCCACCGATTTC
2400____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_450____>
4210 4220 4230 4240 4250 4260
CTGAGGCAGCGGGCCACAGGGACACCTTGTACACGATGCTGATAAAAGTGGGTCAACAAAA
GACTCCGTCGCCCCGGTGTCCCTGTGGAACATGTGCTACGACTATTTCAACCCAGTTGTTTT
2460____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_510____>
4270 4280 4290 4300 4310 4320
CCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTGGAGACGCTGGGAGAGAGAC
GGCCCGCTCTACGGAGACAGGTGTGGGACGACCTACGGAACCTCTGCGACCCCTCTCTCTG
2520____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_570____>
4330 4340 4350 4360 4370 4380
TTGCCAAGCAGAAGATTGAGGACCACTTGTGAGCTCTGGAAAGTTCATGTATCTAGAAG
AACGGTTCGTCTTCTAACTCCTGGTGAACAACCTCGAGACCTTTCAAGTACATAGATCTTC
2580____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_630____>
4390 4400
GTAATGCAGACTCTGCCATGTCCTAA-
CATTACGTCTGAGACGGTACAGGATT-
26_1 TO 2663 OF KILLER(____>

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US98/14495
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 15/00, 15/10, 15/12, 15/63

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 455, 462, 463, 320.1, 325; 536/23.1, 23.5, 24.31; 530/350, 387

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, APS

search terms: death domain, killer, apo 2, tumor necrosis factor receptor, apoptosis, p53, inducible, transgenic, TRAIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADERKA, D. The Potential Biological and Clinical Significance of the Soluble Tumor Necrosis Factor Receptors. Cytokine & Growth Factor Reviews. 1996, Vol. 7, No. 3, pages 231-240, see entire document.	1-20
A,P	US 5,763,223 A (WILEY et al.) 09 June 1998, see entire document.	1-20
A	WO 97/18323 A3 (ICOS CORPORATION) 22 May 1997, see Abstract.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 SEPTEMBER 1998

Date of mailing of the international search report

28 OCT 1998

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INTERNATIONAL SEARCH REPORT

International application No.

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A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/69.1, 455, 462, 463, 320.1, 325; 536/23.1, 23.5, 24.31; 530/350, 387